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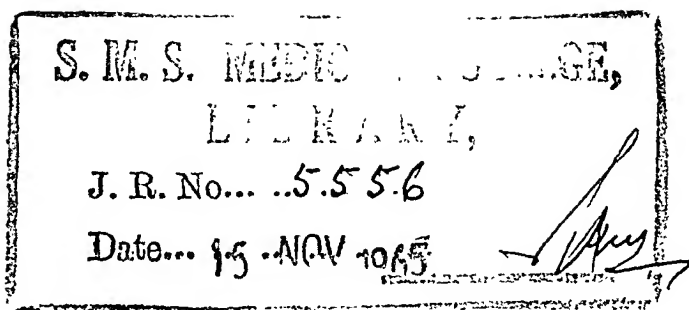
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Erratum.

Vol. 14 P. 289 line 10 of the Summary, for "The ratio RNA/DNA shows" read "The concentrations of RNA and DNA show".

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From the Department of Physiology, University of Lund.

The Effect of Enterogastrone on the Gastric Secretion of the Cat Stimulated by Continuous Administration of Histamine.

By

BÖRJE UVNÄS.

Received 29 September 1947.

It is an established fact that neutral fats inhibit gastric secretion when reaching the duodenum and the upper part of the jejunum. FENG, HOU and LIM (1929) showed on dogs with autotransplanted and consequently denervated subcutaneous pouches that the inhibitory mechanism was mediated by the release of an inhibitory hormone, enterogastrone, from the intestinal mucosa. LIM and co-workers (1930, 1933), IVY and co-workers (1937, 1946) and others extracted from the duodenal and upper jejunal mucosa of hogs a material that inhibits the gastric secretion in dogs. The active principle is claimed to be identical with enterogastrone. The preparations also inhibit gastric motility. Whether the inhibitory effects are due to one or two agents is still obscure (IVY *et al.* 1947). The enterogastrone preparations are considered to contain still another active factor — “an ulcer preventing principle”. This agent is claimed to reduce the ulcer frequency of Mann-Williamson dogs as well as favour ulcer healing in man (IVY 1944, GREENGARD *et al.* 1946). There seems to be no relationship between the secretory-inhibitory and the ulcer-healing effect of the enterogastrone material (IVY and co-workers 1944, 1946).

GRAY, BRADLEY and IVY developed a biological assay method for enterogastrone. Dogs with total stomach pouches were used. A moderate gastric secretion was obtained by “continuous” administration of histamine. It was injected subcutaneously in small

doses every 10 minutes. Enterogastrone was given intravenously and its activity calculated in dog units. A dog unit is defined as the quantity of enterogastrone that on a medium sized dog (10—12 kg) causes a reduction of the secretion of HCl to 50 %. These authors reported that the inhibitory effect of enterogastrone was seen within 10 minutes after the injection and lasted for 2—4 hours. GREENGARD *et al.* (1946) slightly modified the assay method. A total pouch dog was given 0.5 mg histamine subcutaneously and the total secretion during the following 2 hours was measured. A new histamine dose and enterogastrone intravenously was then given. The quantity of enterogastrone that reduced the HCl output during the second secretory period to 50% was said to contain a dog unit.

According to BJÖRKMAN *et al.* (1943) it is possible to obtain a practically constant gastric secretion in cats by continuous intravenous infusion of histamine. It was therefore considered to be of interest to investigate the effect of enterogastrone on the cat's gastric secretion elicited in this way.

Experimental.

The experiments were performed on cats under chloralose-urethane anaesthesia (50 mg chloralose and 500 mg urethane per kg). The gastric juice was collected by a cannula inserted into the stomach (UVNÄS 1945). The volume, and the free and total acidity of the juice were determined in 15 minutes periods of secretion. Titrations were made with N/10 NaOH and Topfer's solution and phenolphthalein as indicators. The histamine was injected continuously intravenously, and concentrations yielding a small or moderate secretion were used. Enterogastrone was given intravenously.

Enterogastrone¹ prepared and assayed according to GREENGARD *et al.* (1946) was used.

Results.

Four different enterogastrone preparations were tested in 15 experiments. In none of these experiments was any inhibitory effect observed. Neither the volume, nor the acidity decreased. On the contrary a distinct potentiating effect was almost always seen after the administration of 10 to 250 mg of the material. The secretory outflow increased but the acidity remained unchanged.

¹ Enterogastrone material assayed for its inhibitory potency was generously supplied by Dr. Greengard as well as by the Upjohn Company. Kalamazoo. Mich.

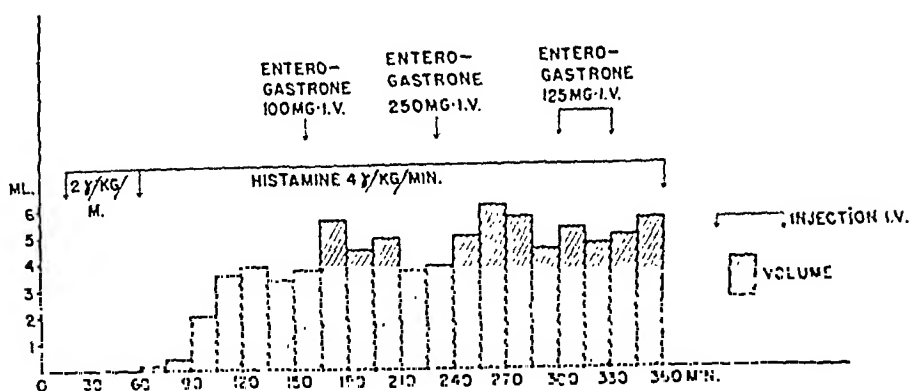


Fig. 1. Cat 3.0 kg. Gastric secretion in ml per 15 minutes under continuous histamine infusion. The effect of enterogastrone administered intravenously.

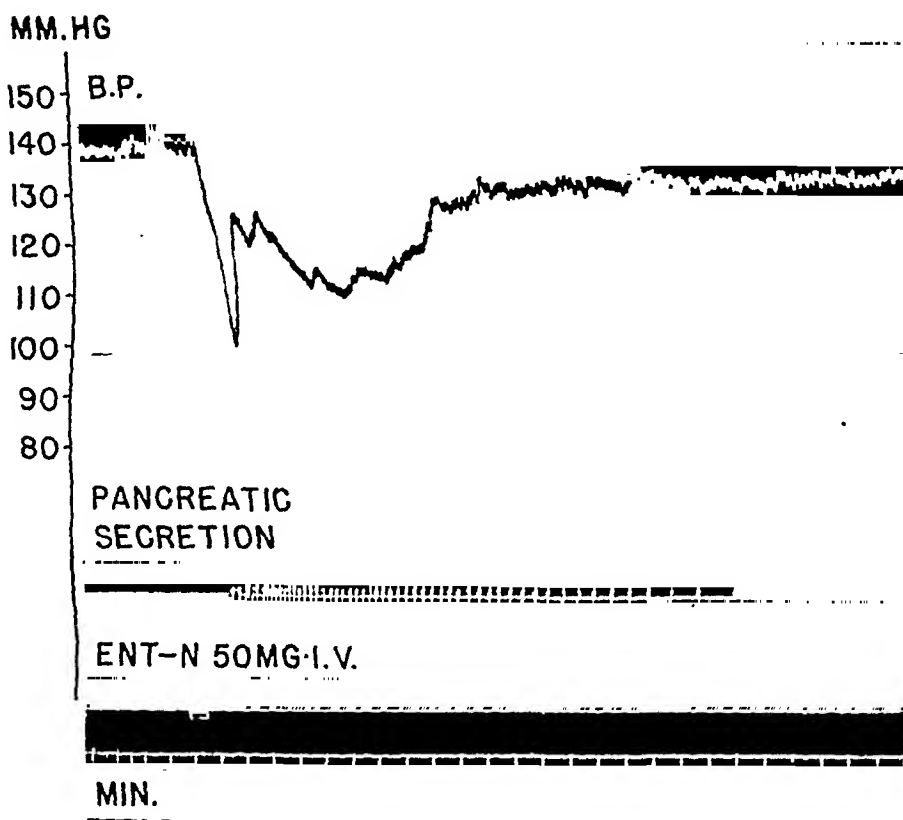


Fig. 2. Cat 3.2 kg. Effect on blood pressure and pancreatic secretion by injection of 50 mg enterogastrone intravenously.

The results were the same regardless of which secretory rate was chosen between 1—5 ml per 15 minutes.

One experiment is shown in figure 1. A moderate secretion was

obtained by the continuous administration of 4 γ histamine di-chloride per kg body weight and minute. The injection of 100 mg enterogastrone material (2 dog units) caused a distinct increase of the secretion that returned to the basal level in 45 minutes. A second enterogastrone injection of 250 mg gave a similar response. Continuous intravenous injection of 150 mg enterogastrone failed to cause any inhibition. Possibly there was a slight increase.

Enterogastrone material injected alone did not cause any gastric secretion. Large doses (50 mg and more) caused a short lasting contraction of the stomach. The increase of the secretory outflow was not, however, due to this phenomenon as the increase lasted for 30—45 minutes and was observed also after small doses that did not affect the gastric motility.

The preparations contained vasodepressor acting material (fig. 2) but apparently no histamine since it did not contract the guinea pig ileum as does histamine. The content of secretin was rather high as the pancreatic secretory response to enterogastrone amounted to about a tenth of that elicited by a commercial secretin material (Pancreotest, Astra).

Comments.

Enterogastrone material found to cause a pronounced inhibition of the gastric secretion in total stomach pouch dogs does not inhibit the histamine-induced gastric secretion of the cat. The reason for this difference between the two species is obscure. It might be due to fundamental differences in the gastric secretory mechanism of the dog and the cat. It is observed for instance by ROTH and IVY (1944) that caffeine stimulates the gastric secretion in cats but not in dogs. On the other hand in the cat the inhibitory effect of the enterogastrone material might be counteracted by concomitant impurities. As observed the preparations contain vasodepressor acting material as well as secretin. KOMAROV (1942) as well as UVNÄS (1945) have observed a secretory effect of preparations from the duodenal mucosa, the secretory agent possibly being gastrin. As the preparation of enterogastrone involves the precipitation of the active material from acid duodenal extracts with sodium chloride, tannic acid, picric acid and acetone, a contamination with gastrin should be suspected. A small

gastrin contamination could possibly explain the lack of inhibitory potency as well as the small potentiating effect of the material.

KOSAKA and LIM obtained an inhibitory principle from HCl-extracts also of the colon mucosa. As no humoral inhibitory mechanism is known to be mediated by the presence of fat or other substances in the colon a further purification of the enterogastrone material and continued investigation of its inhibitory action on different animal species is necessary to establish the identity of the inhibitory agent with the physiologically occurring inhibitory substance.

Summary.

Enterogastrone prepared according to GREENGARD *et al.* does not inhibit the gastric secretion of the anaesthetized cat stimulated by the intravenous infusion of histamine.

These experiments were started during my stay with Dr. A. C. IVY, the Department of Physiology, Northwestern University, Chicago, Ill., U. S. A. I am greatly indebted to Dr. Ivy for his kind interest and great generosity during the months I worked in his laboratory.

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The Effect of Atropine, Scopolamine and some Related Synthetic Drugs on the Insulin Induced Gastric Secretion of the Dog.

By

GUNNAR STRÖM and BÖRJE UVNÄS.

Received 29 September 1947.

As observed already by PAVLOV and his associates atropine inhibits the gastric secretion induced by electrical stimulation of the vagi, by sham feeding, or by a meal. It inhibits the gastric as well as the intestinal secretory phase (SAVICH and ZELIONY 1911—12, IVY, LIM and MCCARTHY 1925, WEBSTER and ARMOUR (1932). In high doses atropine considerably depresses the secretory effect of histamine (KEETON, LUCKHARDT and KOCH 1920, GRAY 1937, ATKINSON and IVY 1937). As histamine is considered to elicit gastric secretion by a direct action on the perietal cells atropine "besides its autonomic blocking effect must act on the secretory cells" (ATKINSON and IVY).

The reports on the effects of therapeutic doses of atropine and belladonna preparations on the gastric secretion of man are conflicting. Some investigators find a decrease of both volume and acidity, others find the volume reduced but the acidity unchanged or even raised and many report no significant change of the secretion. Among recent investigators NYMAN (1943) observed that atropine possessed a considerable inhibitory effect on the histamine induced gastric secretion of man.

In recent years several synthetic substitutes for belladonna alkaloids have appeared on the market. Numerous investigations dealing with their spasmolytic action, and a few reports on their influence on the salivary secretion have been published.

Scarcely any have dealt with the effect on the gastric secretion. ATKINSON and IVY (1937) studied the inhibitory potency of atropine, novatropine and methatropine on the secretion of Pavlov pouch dogs and on man. The gastric secretion was evoked by a meal or the subcutaneous injection of histamine. They found atropine to be as potent as any of its substitutes. NYMAN (1943) found methyl scopolamine to be the most potent inhibitor of the salivary secretion so far studied. After the beginning of our experiments a paper dealing with the effect of spasmolytic drugs on gastric secretion appeared (NECHELES and co-workers 1944). On Pavlov pouch dogs trasentin H was observed to be a potent depressant drug.

Insulin evokes a gastric secretion on dog and man by causing a hypoglycemia that activates "vagal" centers in the brain (LA BARRE and CESPEDES 1931). The juice obtained is of high acidity and high peptic activity. By insulin it is possible to get a vagally induced gastric secretion without the interference of the different secretory phases. In our experiments we therefore used insulin as the secretory excitant.

Experimental.

On three gastric fistula dogs we tested the inhibitory effect of atropine sulphate, homatropine hydrobromic. cryst. (Merck), methyl scopolamine nitrate (Pharmacia), scopolamine hydrobromic. (Leo), ¹syntropan (Roche) and ²vagospasmyl (Leo).

The dogs were starved for 24 hours. The drug under investigation was given intramuscularly 15 minutes before the administration of insulin. This was injected intravenously in a dose found to evoke a moderate gastric secretion. The volume of the secretion was measured in 15 minute periods, the acidity determined by titration against N/10 NaOH using dimethyl-azobenzol and phenolphthalein as indicators for free and total acidity; whilst the peptic activity was measured by the modified Anson and Mirsky method described by URXÄS (1945).

The response to insulin varies considerably from dog to dog. A dose between 0.1 and 0.5 units per kg body weight usually evokes a moderate secretion. The secretory response of a particular dog is rather constant from day to day but over a longer period the sensitivity to insulin shows spontaneous changes. Frequent control experiments are therefore necessary. Experiments were performed on a particular dog 2 or 3 times a week, every third or fourth experiment was used as a control.

¹ Tropaic ester of 3-diethyl-amino-2, 2-dimethyl-propanol.
² Diphenyl-acetyl-diethyl-amino-ethanol.

To eliminate as far as possible the influence of spontaneous variations, our "normal" values were calculated as the mean value of the control experiment preceding and following the test experiment.

Results.

The secretory response to insulin usually started within 15 to 30 minutes, reached a peak in about an hour, then gradually declined and reached the initial level in about two hours. The acidity was high, varying from dog to dog from 120 to about 150 milliequivalents per litre. The peptic activity was also high, usually amounting to about 50—60 peptic units per ml.

Table.

Drug	Number of experiments	Secretion in % of normal (mean value)
Atropine		
γ/kg		
1.4	6	49
7.2	4	16
14.3	3	3
36.0	1	7
Scopolamine		
γ/kg		
0.36	1	100
0.72	1	41
3.6	2	24
7.2	2	23
10.7	1	14
Methyl scopolamine		
γ/kg		
0.072	1	94
0.18	2	73
0.36	2	43
0.72	1	19
3.6	2	0
7.2	2	0
Homatropine		
Mg/kg		
0.072	1	61
0.36	2	45
0.72	3	3
Syntropan		
Mg/kg		
1.4	1	97
3.6	5	59
7.2	1	48
10.7	2	14
14.3	2	11
Vagospasmyl		
Mg/kg		
0.36	1	100
1.8	2	47
3.6	4	48
7.2	3	40
14.4	1	6

All the investigated drugs inhibited the gastric secretion. Moderately inhibitory doses depressed the secretory volume but did not significantly change the acidity. Higher doses depressed both the secretory rate and the acidity. The peptic secretion seemed to be inhibited in the same degree as the acid secretion. The peptic activity remained unchanged until very high doses of the drugs were given. It then declined as did the acidity.

In the table the summarized results from 59 experiments are given. The inhibition of the gastric secretion is calculated from the total amount of HCl secreted during the two hours following the insulin injection. The secretion is calculated as per cent of "normal".

Comment.

Methyl scopolamine is the most potent gastric secretory depressant of the drugs investigated, about 5 γ per kg body weight giving a complete inhibition of the secretion. It is slightly more potent than scopolamine and atropine, both of which inhibit the secretion to about the same degree. Homatropine is a weak inhibitor. The spasmolytic drugs syntropan and vagospasmyl depress the gastric secretion only if very high doses are given. In fact syntropan in doses of 7.2 mg per kg body weight or more regularly caused an abundant salivary secretion, muscular weakness, and signs of nausea.

Summary.

Methyl scopolamine is the most potent gastric secretory depressant of the atropine substitutes so far studied. The spasmolytic drugs syntropan and vagospasmyl depress the gastric secretion only if given in very high doses.

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Recording Devices in Connection With Geiger—Müller Counters.

By

THORALF KYRRE.

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In the recent years, after HEVESY's pioneer work, the artificially produced radioactive isotopes of the elements have been used more and more in biological researches.

These radioactive, or labelled, atoms employed as tracers make quantitative analyses of extremely small amounts of material possible (of the order of 1×10^{-6} milligrammes, or even less).

The radioactive isotopes have formerly been difficult to get, but as they are now being produced in ample quantities as by-products in various atomsplitting experiments carried out for some other purpose, they may be expected to become more easily obtainable for such experiments as those forming the subject of this paper.

It was difficult, too, to procure the Geiger—Müller tube, the function of which is to respond to the emission of β -particles so that each β -particle striking a "window" in the tube will cause an impulse of current to be generated in the latter. These G—M tubes are now being manufactured commercially in various countries; it is still prohibited, however, to export this article from the U. S.

Since the G—M tubes, the counting devices used in connection with them, and their applicability thus seem to be under-

As the detailed diagrams cannot be expected to be of general interest, they are not given here; they can be ordered, however, from the author who is also willing to build the apparatuses mentioned in compliance with requirements. Address: Box 12035, Stockholm 12.

going a rapid development, it is the author's opinion that the present article — in which various methods of measuring radioactivity are mentioned, and a description given of the apparatus designed and built by the author for The University of Aarhus Institute of Normal Anatomy, and The Radium Station of Aarhus, in Denmark — may be of interest.

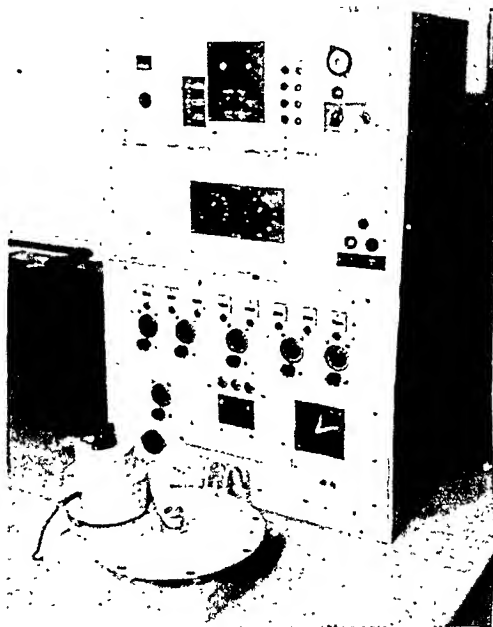


Fig. 1. A recording device and regulated 2,000-volt power supply for Geiger-Müller tube and an automatic machine capable of conveying up to 8 preparations successively under the G—M tube. (Normal Anatomisk Institut, Universitet, Aarhus, Denmark.)

Recording of Impulses.

The β -particles emitted from the active preparation arrive at intervals of unequal length, and very frequently two or more impulses follow each other in such rapid succession that an ordinary telephone subscriber's meter cannot keep pace with them. There are 4 methods of avoiding the resulting error:

1) A *theoretical-statistical correction* (HOLM-JENSEN 1943) can be applied, the calculation of which, however, makes it necessary to know the reaction time of the counter; the reaction time is not constant and cannot easily be determined.

This method was therefore abandoned when the newer methods mentioned below had been developed.

2) A *scaling device* can be introduced, causing only a certain fraction of the impulses to be recorded by the counter.

3) A *registering device* can be employed to store a succession of received impulses temporarily.

4) An *integrator* can be used which, in connection with a pointer-instrument, indicates directly the number of impulses received per minute.

Scaling Device. This is called "scale of n ", n standing for the ratio between generated and counted impulses. When a small

n is chosen, f. ex. 2 or 4, the scaling device will be defective in that inaccuracy may become considerable if the preparation is too intensely radioactive, in which case n impulses may arrive within a period that is shorter than the reaction time of the meter. When, on the other hand, a higher n is chosen, e. g. 8, 10, or 16, the possible error of the count will be $n-1$ as the number of impulses received by the scaler since the counter advanced last is unknown. This inexactness becomes particularly important when dealing with preparations of low intensity. An arrangement for operating the scaler with different values of n by means of a switch can be introduced by way of remedying this drawback, supplemented with neon lamps, or a pointer instrument, to indicate the number of residual impulses in the scaler. These auxiliary devices, however, are not immediately available in cases where several preparations are to be conveyed automatically into the count chamber, one by one, those of high intensity alternating with those of low intensity, and background counts being taken between for control (AMBROSEN, 1942).

Registering Device. The register picks up the impulses as fast as they are being received from the G—M valve, but transmits them to the meter at a certain maximum rate of speed, adapted to the reaction time of the meter; the impulses are stored by the register until the meter can receive them, just as a sudden rush on a booking office will result in the forming of a queue because the tickets can only be sold at a certain maximum rate of speed. The author has tried this method experimentally, but not employed it in practice as yet. The intensity of the preparation is limited by the condition for the application of this device, viz. that the average of impulses must be less than the maximum rate of count at which the meter can work accurately. The maximum rate for a telephone subscriber's meter is about 25 counts per seconds, i. e. 1,500 impulses per minute. Preparations of such high intensity are scarcely likely to be used in biological research.

The idea of replacing the ordinary subscriber's meter with a faster type of meter suggests itself. Such a fast-going meter was constructed by the author by means of an ordinary clock movement from which the balance was removed; the anchor was then connected mechanically to the armature of a polarised telegraph relay, the mainspring was taken away, and an electric motor was coupled to the movement via a friction clutch. The windings of the relay were connected to the

anode circuit of the last scaling stage. Any one impulse occurring in this circuit would energise the telegraph relay, causing the escapement wheel to move one step. Consequently the hands (seconds and minute hands) would advance for each impulse produced. The described meter responded easily to 200 c. p. s. Such a meter would be too expensive in practice, and the possibility of faulty working is greater than in the case of the telephone meter.

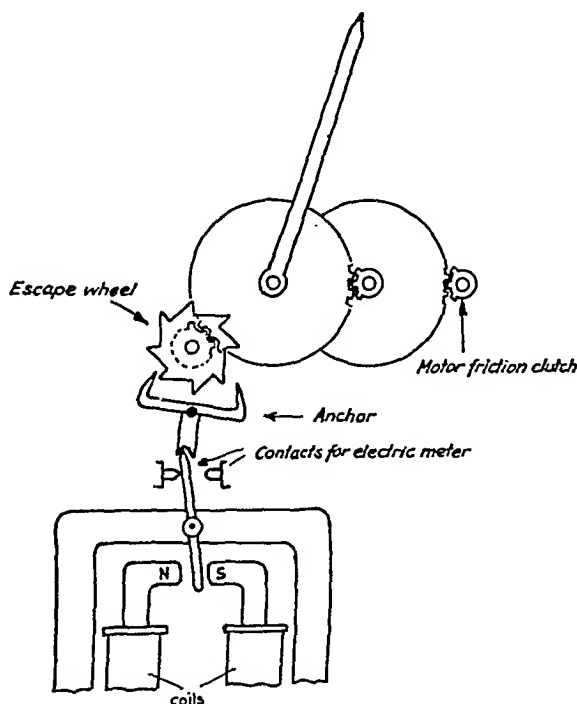


Fig. 2. Fast-going counter for max. 200 c. p. s. constructed by means of an ordinary clock movement connected mechanically to the armature of a polarised telegraph relay.

Integrator. For radioactive preparations of a certain minimum intensity, the average number of impulses per minute can be recorded on a pointer instrument. If below this minimum, the preparation will make the pointer waver too much to give any accurate reading. The turning-point is at about 100 impulses per minute. The principle of the integrator is the following: Each impulse produced will cause a certain quantity of electricity to be pumped into a capacitor which is being continuously discharged by a constant flow of current through a high resistance to a high negative potential. The instantaneous potential across the capacitor is indicated on a thermionic voltmeter; this voltage will be proportional to the ratio between the quantities of electricity fed

to, and led away from, the capacitor and thus also proportional to the number of impulses per unit of time (GRAVES, 1947). The integrator is particularly useful when working simultaneously with the electromagnetic meter, thus partly being a means of control and partly affording a first-hand estimate of the intensity of the preparation. On the basis of this estimate it is decided how long the preparation must be measured in order to get sufficient accuracy. The pointer instrument may be replaced with a graphic instrument in which a paper chart rotates with constant speed; the obtained curve is very accurate, when the decay factor is allowed for. The same arrangement can be used instead of a series of meters by letting the automatic substitution of the next preparation for the preceding one coincide with the paper chart being shifted one step forward. On account of its deficient performance when counting preparations of low intensity, the author did not use the integrator in his apparatus.

Automatic Changing of Preparations. It is usually desirable to measure several preparations in immediate succession of each other in order that their respective intensities may be compared. Different preparations may require counting periods of different length and for low-intensity preparations, the background count must be taken into consideration so that it is necessary to take pure background counts between. A mechanism for feeding the preparations into the count chamber according to a predetermined schedule would be useful for this purpose, and an electrically operated automatic machine, affording more combinations than previous constructions, was therefore designed and built. The conveyor is a circular aluminium disc with 8 bored holes placed along its circumference so as to be equidistant. Placed in aluminium cups in the holes the preparations are, one by one, conveyed into the count chamber by turning the disc. The rotating force is imparted to the disc by an electric motor, through a worm gear, and a special clamping device arrests the disc so that all preparations will be placed in exactly the same position under the count chamber. The worm is released from the worm wheel of the disc after the changing operation, to permit the disc to be turned by hand also. The disc shaft runs in two tight-fitting ball bearings and the disc is precision-made, the variation in vertical distance between the preparations and the count chamber being not over a few hundredth of a millimeter. The G—M valve is enclosed in a cylinder of lead which protects it against

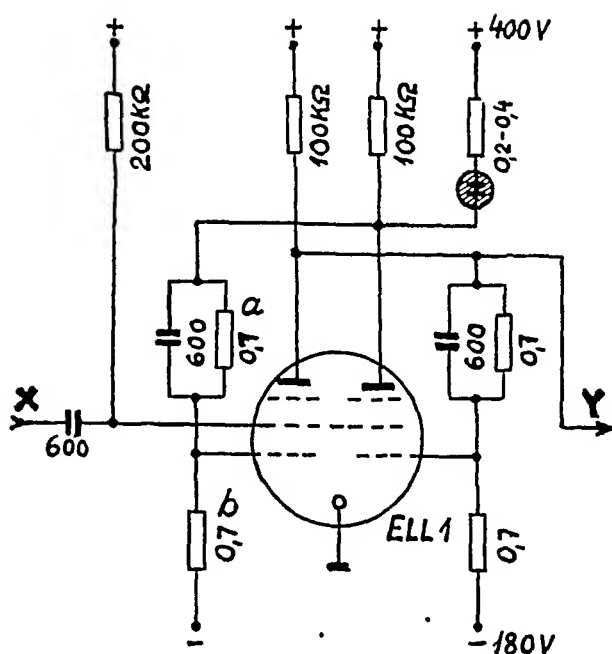


Fig. 4. The duoscale. If a number of impulses enters at x only half this number will leave at y . Several scaling stages can be coupled after one another.

of 10^{-5} sec.) of the produced impulse. As pre-amplifier were used the two triodes in the combination valve EDD11 which are coupled so as to form an ordinary two-stage amplifier. A 2 mV impulse from the G—M valve will cause the pre-amplifier to transmit an impulse of 50 volts to the input of the scaler, which voltage is sufficient to control the latter; a higher input voltage will cause overmodulation of the second grid so that the impulse transmitted does not exceed about 70 volts. A continuous discharge will start in the G—M tube if the power supply is regulated to a too high voltage. The existence of an extinction valve in the circuit automatically limits the flow of current in the G—M tube. In both cases, however, the grid voltage of the first valve will fall, at the same time causing the potential across the anode resistor to drop. The neon lamp connected in parallel with the anode resistor will then be extinguished, thereby indicating that the power supply to the G—M tube is of too high voltage. In borderline cases each impulse will cause a flash-over so that glows can be observed in the neon lamp. Normal impulses are of such short duration that the glows are unperceivable.

Scaling Stages. The function of each scaling stage is to transmit half the number of impulses it receives. If n scaling stages are coupled after one another, $\frac{1}{2^n}$ of the impulses received will be transmitted (JACOBSEN and SIGURGEIRSSON, 1943).

In the coupling of the double pentode ELL1 shown above, the main

part of the anode current will flow either in one or in the other system. A negative impulse sent to the common screen grid of the two systems will check the anode current flowing in the open system, with the result that a positive impulse is transferred from the anode of one system to the control grid of the other system, and the coupling capacitor will be charged. Owing to this charge, the control grid of the other system will have the highest potential when the screen grid has recovered its normal voltage, and the anode current will consequently flow in this system. Thus, if a number of impulses enters at x , only half this number will leave at y .

The author made some alterations in the formerly employed methods of coupling (AMBROSEN, NIELSEN, and SIGURGEIRSSON, 1942). Thus, the cathode was connected directly to earth which resulted in better stability. A high negative grid bias was chosen (-180 volts) in consequence of which the resistor b would be given a high value ($a = b = 0.7$ megohm) so that half the variation in anode voltage of one system becomes transferred to the grid of the other system. The grid voltage of the positive grid will immediately fall to about 10 volts owing to a grid current beginning to flow. High anode resistors (100 kilohms) and a high anode voltage (400 volts) were chosen in order to obtain considerable variations in the anode potentials.

The variations in voltage and current in the two systems when open and closed are as follows:

	Open	Closed
Anode voltage	10 V	300 V
Anode current (plus grid current to the other system)	4 mA	1 mA
Grid voltage	+ 10 V	- 70 V

In order to make it possible to ascertain at any given moment the "position" of the valve, *i. e.* which circuit is open, and which is closed, a neon lamp with series resistor was connected in parallel with the anode load of one of the valve halves. In the case of a closed circuit, the voltage drop through the anode resistor is too small (100 volts) to light the lamp, whereas, in the case of an open circuit, the drop (390 volts) exceeds the ignition voltage (about 180 volts), and the neon lamp becomes lighted.

Scale of 10. Scalars are usually designed in such a way that there is a ratio of $2n$ between the numbers of impulses received and transmitted, n standing for the number of scaling stages. In biological research the radioactivity is generally of such low intensity that a scale of 8 is sufficient. The result as read on the meter must then be multiplied by eight. To avoid this inconvenient multiplication factor, which is likely to cause errors and waste of time when many readings are to be taken, the author introduced a scale of 10.

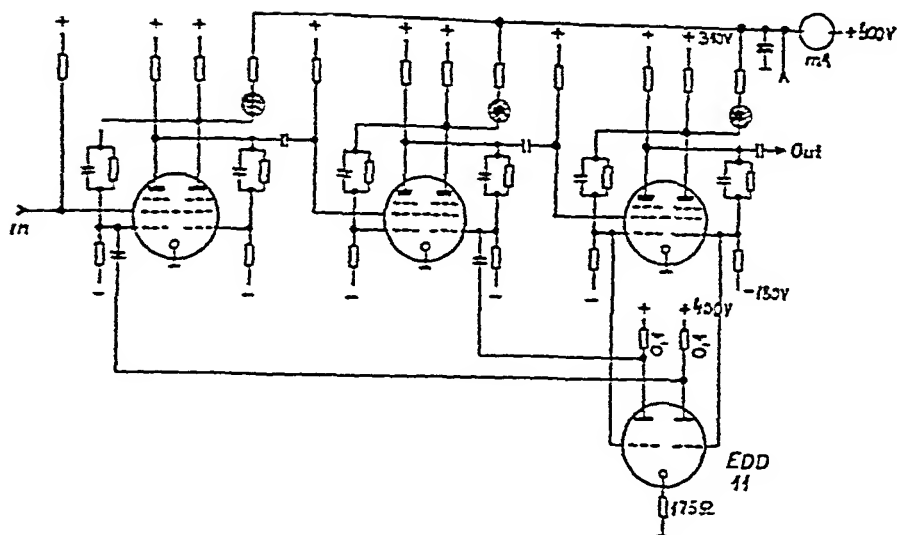


Fig. 5. The scale of 5. A scale of 5 is a scale of 8 (3 scaling stages coupled after one another) with a "feedback", the effect of which is that 3 "positions" are omitted. One additional stage will execute another halving, the result being a scale of 10.

In order to build a scale of 10 it is necessary to start with a scale of 5; one additional stage will execute another halving, the result being that every tenth impulse only will be recorded.

A scale of 5 is in the principle a scale of 8 provided with a kind of "feedback", the effect of which is that 3 "positions" are omitted: let o and x denote the two possible "positions" of the individual stages, o being the starting position, and let it be the change from position x to position o that influences the next stage; the succession of events in a scale of 8 can then be expressed as follows:

Scale of 8.

Impulse no.	1st stage	2nd stage	3rd stage
0	o	o	o
1	x	o	o
2	o	x	o
3	x	x	o
4	o	o	x
5	x	o	x
6	o	x	x
7	x	x	x
8	o	o	o

The "feedback" making the scale of 8 skip three stages will appear from fig. 5. When the 3rd stage changes from position o to position x , an impulse is sent back to 1st stage, causing the latter to change from position o to position x ; simultaneously, an impulse goes to 2nd stage

which, also, changes from position o to position x. This means that the scaler changes directly

from positions	x	x	o
to positions	x	x	x

or, cf. the table above, the positions corresponding to impulse nos. 4, 5, and 6 are missed out. The "feedback" will function again when the last stage changes from x to o, this time trying to make 1st and 2nd stage change from x to o. This change has already occurred, however; 1st and 2nd stage are in position o, and no new change will take place.

In tabular form the succession of events in the scaler is now the following:

Impulse no.	1st stage	2nd stage	3rd stage
0	o	o	o
1	x	o	o
2	o	x	o
3	x	x	o
4 (unstable, changes to:)	o	o	x
	x	x	x
5	o	o	o

The valve EDD11 only serves to prevent changes occurring in 1st and 2nd stage from influencing 3rd stage, and is not necessary for the purpose of amplification.

Finally, the scale of 5 is converted into a scale of 10 by means of the additional 4th stage, coupled in the usual manner to the 3rd stage.

Output Stage. The last scaling stage must be coupled to the telephone meter in such a way that the latter will take one step forward when the former changes from position x to position o. The scaler input will then have received 10 impulses since it was in the starting position.

Formerly, an amplifying valve, a thyratron valve, or a multi-vibrator was placed between the last scaling stage and the meter. The author made the last scaling valve operate the meter via a polarised telegraph relay, a special power valve such as mentioned above thus being unnecessary.

As the resistance of the anode load of the last valve is 12 kilo-ohms, the anode current will be large enough to work the telegraph relay. The polarised relay is of the two-coil type; the coils are connected in series with the two anode resistors. A passage of current in one coil will make the armature move to one side, and when the other coil is energised the armature will move to the other side.

The values of the anode voltage and current. voltage and current will be the following:

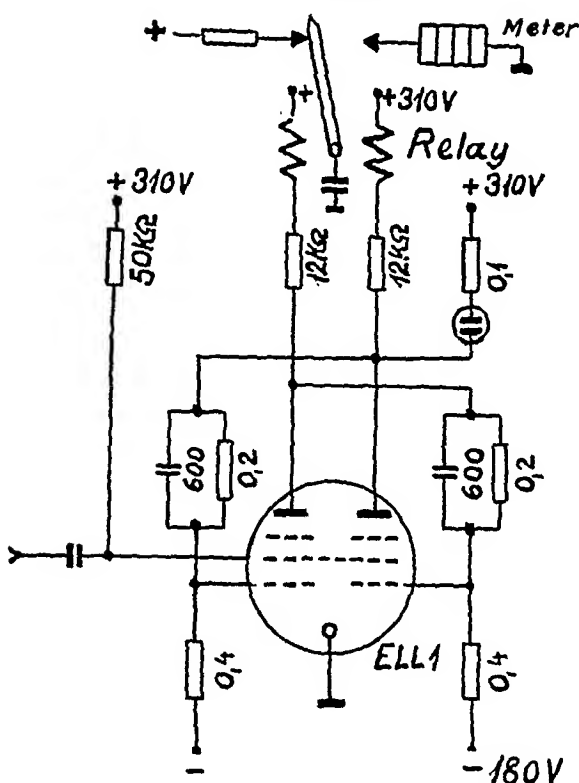


Fig. 6. Output scaling-stage. The last scaling tube operates the counter via a polarised telegraph relay. The coils of the relay are connected in series with the two anode resistors.

Last Scaling Stage.

	Open	Closed
Anode voltage	100 V	370 V
Anode current (plus grid current to the other system)	25 mA	2.5 mA
Grid voltage	+ 10 V	— 65 V

Reliability and quick action can be counted upon as a current of 8 milliamperes is sufficient to energise the telegraph relay.

The armature of the relay will click over for every fifth impulse. In one position a circuit is closed, charging a capacitor through a series resistor; in the opposite position another circuit is closed so that the same capacitor is discharged through the windings of the meter, causing the latter to take one step forward. The advantage of this form of supplying energy to the meter is that a momentary application of a considerable over-voltage will make the meter respond quickly without unduly heating the coil. The meter is deenergised very rapidly as the capacitor

is completely discharged even before the armature reaches the pole face.

The same telegraph relay can be used to operate a "clock movement meter" simultaneously, in the manner described earlier in this article.

Indications of Units. By the described arrangement, only every 10th impulse will be recorded on the meter. For the purpose of making it possible to read the units also, the respective neon lamps of the four stages were marked with the figures "1", "2", "1", and "5". The sum obtained by adding the values affixed to the lighted neon lamps is equal to the number of units that should be added to the tens indicated by the meter.

If the series resistors of the neon lamps be given such values that the passing currents, when the lamps are lighted, are proportional to the affixed numerical values, and if the total current be sent through a milliammeter giving full deflection when all lamps are lighted, the deflection will indicate the units provided that the scale of the instrument has been marked out in 9 intervals or degrees.

The pointer will move forward by degrees, one for each impulse received from the count chamber, until the 9th degree; from there it will jump back to zero, and start over again. Watching the movements of the pointer while the scaler is working gives a good control of its accurate functioning.

The capacitor connected to a point between the instrument and the neon lamps serves to prevent the separate stages from falsely influencing each other.

Testing Device. The correct working of the scaler can be checked by feeding a 50 c. p. s. alternating current from the mains to the input of the scaler via a transformer. The meter should then count at the rate of 5 units per second.

Another testing device, designed to make the scaler advance one step when a pushbutton was pressed, was also made; a small capacitor across the scaler input was discharged by pushing the button. This arrangement caused the author unforeseen trouble, however, as the scaler would record several impulses instead of one, the reason being that a spark discharge occurred in the moment of contact-making so that several successive contacts were established instead of one, and thus the scaler input received several impulses. In spite of various attempts at getting round this problem, the author never succeeded in making the device

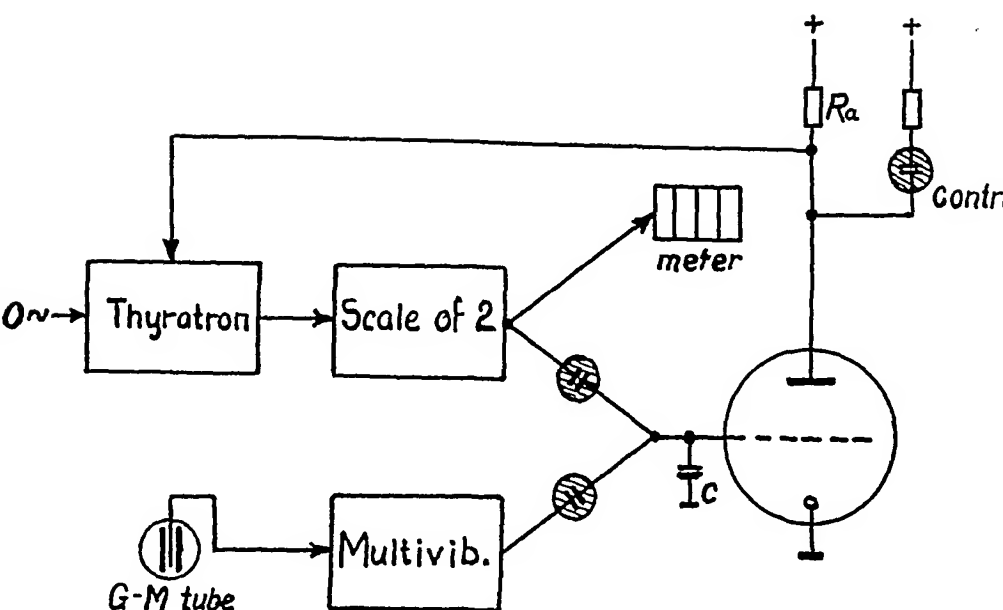


Fig. 7. Registering Device. The register picks up the impulses as fast as they are being received from the G—M tube, but transmits them to the counter at a certain maximum speed, adapted to the reaction time of the counter.

work quite reliably; on the other hand, the phenomenon was plain evidence of the fact that an ordinary closing of a circuit over a pair of contacts actually consists of a succession of contact-makings which could be recorded in this particular case, owing to the short reaction time of the scaler.

Registering device. The schematic diagram above shows an arrangement which was tried experimentally by the author. A negative impulse originating from the G—M valve is amplified and sent through the multivibrator, the function of which is — regardless of the character of the incoming impulse — to deliver an impulse of constant amplitude and duration. The peak of the produced impulse will pass the neon lamp and convey a negative charge to the capacitor C, causing the anode current to diminish and the anode voltage to increase. This, in turn, will cause the thyatron circuit to open and the mains a. c. (50 c. p. s.) to be fed to a scale of 2 which, besides, acts as a multivibrator and, as such, will send positive impulses of the same amplitude and duration as the negative impulse originating from the first multivibrator. The very first of these positive impulses will neutralize the charge of the capacitor, the anode voltage will diminish, and the thyatron will be blocked. Only one impulse from the scaler

has reached the grid; at the same time, however, an impulse has reached the meter and made it advance one step. When the G—M tube sends a limited succession of impulses amounting to not more than 25 per second, the capacitor will maintain a negative charge until the main impulses succeed in neutralizing the capacitor; meanwhile, the meter will be going at its maximum speed of 25 counts per second. In case G—M tube sends impulses at an average rate of more than 25 per second, the anode current will fall to such a value that the neon control lamp will be extinguished. Between the grid and a normal negative grid polarisation voltage supply is placed a high resistance (not shown in the diagram) which serves to keep the grid potential on a fairly normal level.

Summary.

The article gives a survey of various devices for recording the number of impulses received from a Geiger—Müller tube when the latter is exposed to radiation from a radioactive preparation. It contains a description of an automatic machine capable of conveying up to 8 separate preparations successively under the G—M tube, once or several times, and for periods of variable length (from 2 to 60 minutes).

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Aus dem Physiologischen Institut der Universität Helsinki.

Über die Grösse der absoluten und der ihr nachfolgenden Reizschwellen von Momentanlichtempfindungen.

Von

YRJÖ REENPÄÄ.

Eingegangen am 4. Oktober 1947.

Im vorigen Jahre publizierte der Autor dieser Arbeit in dieser Zeitschrift zusammen mit JALAVISTO und NIINI eine Untersuchung über die Gültigkeit und Grenzen der bilinearen Reizausdrücke bei schwellenmässigen Lichtempfindungen. Die Gültigkeit der von BLOCH u. a. gefundenen Schwellenregel $L \cdot t = E_1$, wobei L die Lichtintensität, t die Zeitdauer und E_1 die konstante Energie des Minimalreizes bedeuten, wurde auch von uns erneut bestätigt. Die Regel gilt genau bis zu Zeitdauern von 100 bis 120 σ . Ausser an der absoluten Schwelle untersuchten wir die gegenseitige Abhängigkeit der Reizgrössen Intensität und Zeit auch an drei überschwelligen Empfindungsstellen. Es wurde gezeigt, dass auch an diesen Stellen eine Produktenregel näherungsweise gilt: $L \cdot t = E_m$, wobei E_m die der m -ten Stelle entsprechende Reizenergieflächen-dichte bedeutet. Die Zeitgrenze des Geltens dieser Regeln ist enger als an der absoluten Schwelle. Weiter untersuchten wir, bis zu welcher Zeitdauer der Reize die Lichterlebnisse *phänomenal* als momentan empfunden werden. An der absoluten Empfindungsschwelle erstreckt sich dieses Gebiet bis zur Zeitdauer von 120 σ , bei den überschwelligen Empfindungen bis zu 20—30 σ , ungefähr entsprechend den Grenzen des Geltens der bilinearen Reizausdrücke. Es scheinen also die oberen zeitlichen Grenzen einerseits der Gültigkeit der Produktenregeln und andererseits der phänomenalen Momentanität ungefähr an ein und derselben Zeit-Stelle zu liegen, ein Verhalten, dessen Bedeutung in einer zweiten Abhandlung besprochen wurde (REENPÄÄ, 1947).

Tabelle 1.

Werte der der absoluten Empfindungsschwelle ($m = 1$) und einiger höheren Empfindungsstellen ($m = 4, 10, 20$) entsprechenden Dauerlichtintensitäten (L_m) sowie Momentanlichtreizenergien (E_m). Das Bild der Belichtungsfläche innerhalb der Fovea centralis.

m	L_m (Lux)	E_m (Lux · Sigmen)
1	0.0238	2.35 ± 0.27
4	0.0954	6.01 ± 0.50
10	0.238	13.5 ± 0.98
20	0.476	28.9 ± 3.2

In der beiliegenden kleinen Tabelle 1 ist eine Zusammenfassung der von uns erhaltenen Ergebnisse gegeben. Hier bedeuten L die Lux-Werte der Lichtintensitäten, wenn es sich um Dauerempfindungen handelt. Der erste Wert ist die Intensität der absoluten, der 1. Schwellenempfindung ($m = 1$), die folgenden Werte sind Intensitäten, welche 4, 10 bzw. 20-mal grösser als die 1. Schwellenintensität sind ($m = 4, 10$ bzw. 20). Die Energiewerte E_m sind die aus dem Versuchsmaterial berechneten Mittelwerte der Produkte $L \cdot t$ ($\text{Lux} \times \sigma$), also der Reizenergiewerte der *Momentanempfindungen*, welche phänomenal mit den entsprechenden *Dauerempfindungen* äquivalent sind (dieselbe erlebnismässige Intensität haben). So bedeuten z. B. E_1 die Reizenergie der absoluten Momentanempfindung und L_1 die Reizintensität der absoluten, also phänomenal äquivalenten, entsprechenden Dauerempfindung. Die in der Tabelle 1 angegebenen Energiewerte sind die den Zeitwerten 1 bis 15 σ entsprechenden, berechneten Mittelwerte. Wir haben diese, nur den kleinsten t -Werten entsprechenden E -werte zur Grundlage der folgenden Besprechungen genommen, weil die Konstanz des Produktes $L \cdot t$ hier am besten zu sein scheint. Ein Verwenden der Mittelwerte von $L \cdot t$ bis zur zeitlichen Grenze von 100 σ bei der absoluten Schwelle und 40 σ bei den höheren Schwellen würde das Ergebnis nicht prinzipiell beeinflussen, wie später gezeigt werden soll.

In der Abbildung 1 sind die L - bzw. m -Werte auf der Abszisse und die phänomenal entsprechenden E -Werte auf der Ordinate abgetragen. Die kleinen Kreise geben die in der Tabelle 1 angegebenen E_m -Werte, also die den t -Werten 1 bis 15 σ entsprechenden Werte wieder. Evident liegen die vier Werte sehr gut auf einer Geraden, womit wir die Gleichung $E - E_0 = k \cdot m$ aus-

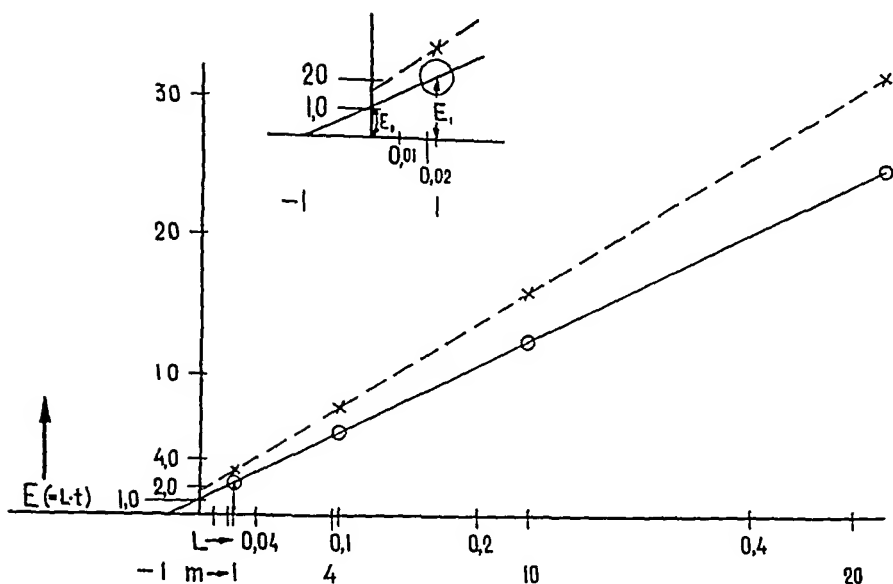


Abb. 1. Abhängigkeit der Momentanlichtreizenenergien (E ; in Lux · Sigmen) und der entsprechenden Empfindungsstellen (m) sowie Dauerlichtintensitäten (L ; in Lux)

stellen können, wo E_0 den linear extrapolierten Wert von E bei dem Wert $m = 0$ sowie k eine Konstante bedeuten. Wenn wir des weiteren berücksichtigen, dass E_0 ersichtlich sehr genau gleich $\frac{E_1}{2}$ ($= 1.18$ Lux) ist, sowie dass auch k gleich $\frac{E_1}{2}$ ist, erhalten wir als rein empirisches Ergebnis die Gleichung $E = (m + 1) \cdot \frac{E_1}{2}$.

Die Kreuze in der Abb. 1 entsprechen den bis zu den t -Werten 100 bzw. 40 σ berechneten $L \cdot t$ -Werten. Ersichtlich liegen diese Werte nicht ganz so gut auf einer Geraden wie die Vorigen. Die Aufstellung der Gleichung der die Kreuze zusammenbindenden Geraden würde aber, wie ersichtlich, zu einer ähnlichen Formel führen, wie wir sie oben aufgestellt haben.

Da die Stellenzahlen m proportional den Lux-Werten L der Dauerreize sind, kann die obige Formel auch in der Form $E = \left(\frac{L}{L_1} + 1 \right) \cdot \frac{L_1}{2}$ geschrieben werden, wo L_1 den Dauer-Luxwert der absoluten Schwelle ($m = 1$) angibt. Wenn zwei um eine Unterschiedsschwelle von einander geschiedene, also zwei nach einander folgende E - bzw. L -Werte mit den Indizes s und $s+1$

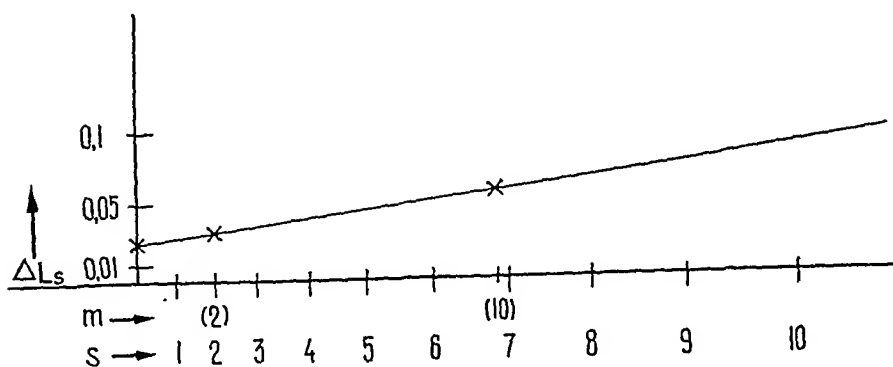


Abb. 2. Graphische Darstellung zur Bestimmung der Ordnungszahlen (S) und der entsprechenden Dauerunterschiedsschwellen (ΔL_s), mit Hilfe der Stellenzahlen (m) und der ihnen entsprechenden Unterschiedsschwellenwerte an den Stellen $m = 2$ u. 10.

bezeichnet werden und weiter die Differenzen $E_{s+1} - E_s = \Delta E_s$ bzw. $L_{s+1} - L_s = \Delta L_s$, so erhalten wir:

$$\Delta E_s = \frac{E_1}{2L_1} \cdot \Delta L_s,$$

welche Formel die gegenseitige Anhängigkeit der Momentan- und der Dauerreizunterschiedsschwellen von einander wiedergibt.

Wir haben früher die Dauerreizunterschiedsschwellen an zwei nahe der absoluten Schwelle liegenden Stellen (an den Stellen $m = 2$ bzw. 10) bestimmt (JALAVISTO, NIINI u. REENPÄÄ). Ausgedrückt als S_{50} -Werte (s. die obengenannte Abhandlung S. 158) sind diese Unterschiedsschwellen an der Stelle $m = 2$ gleich 0,028 Lux und an der Stelle $m = 10$, 0,056 Lux. Als Schwellenwerte sind hierbei die doppelten S_{50} -Werte angesetzt, wie es natürlich erscheint.

In der graphischen Darstellung Abb. 2 sind die Stellenzahlen $m (= 2$ bzw. 10) auf der Abszisse aufgetragen und die beiden entsprechenden Dauerunterschiedsschwellen auf der Ordinate. Auf dieser ist weiter die Dauerreizschwelle bei der absoluten Empfindung angesetzt, welche als die der nullten Stelle nachfolgende Unterschiedsschwelle angesehen werden kann. Diese erste Dauerunterschiedsschwelle hat den empirischen Wert 0,024 Lux (s. die obige Abhandlung S. 153), und wir sehen, dass dieser Wert in die graphische Abb. 2 eingeführt, mit den zwei übrigen Werten (den Werten $m = 2$ u. 10 entsprechend) sehr gut auf einer Geraden zu liegen kommt. In der Nähe der absoluten Lichtschwelle scheinen also die Dauerreizunterschiedsschwellen mit steigender Licht-

intensität bzw. mit steigender Stellenzahl linear zu wachsen, was uns eine Möglichkeit gibt, alle die untersten Dauerreizunterschiedsschwellen interpolatorisch genau zu bestimmen. Dies ist in der Tab. 2 geschehen, in der die den nacheinander folgenden Ordnungszahlen der Unterschiedsschwellen (s) (die Stellen dieser Zahlen sind auch auf der Abszisse der Abb. 2 angesetzt) entsprechenden Dauerunterschiedsschwellen (ΔL_s) sowie die entsprechenden Reizlichtwerte (L_s) angegeben sind.

Mit Hilfe der graphischen Darstellung Abb. 1 oder besser der ihr entsprechenden Gleichung können dann die den obigen Dauerreizwerten phänomenal entsprechenden *Momentanreizwerte* E_s bestimmt werden, wie dies in der Kolumne E_s der obigen Tab. geschehen ist. Die entsprechenden *Momentanunterschiedsschwellen* in der folgenden Kolumne ΔE_s sind aus diesen Zahlen dann durch Subtraktion erhalten worden ($E_{s+1} - E_s = \Delta E_s$).

Tabelle 2.

Die den verschiedenen nacheinanderfolgenden Unterschiedsschwellen (S) entsprechenden Dauergrundreize L_s , Dauerunterschiedsschwellenreize ΔL_s , sowie Momentangrundreize E_s und Momentanunterschiedsschwellenreize ΔE_s .

S	L_s Lux	ΔL_s Lux	E_s Lux	ΔE_s Lux
0	0	0.024	0 (1.2)	2.4 (1.2) = E_1 (ΔE_0)
1	0.024	0.026	2.4	1.3
2	0.050	0.028	3.7	1.4
3	0.078	0.034	5.1	1.7
4	0.112	0.038	6.8	1.9
5	0.150	0.044	8.7	2.2
6	0.194	0.050	10.9	2.5
7	0.244	0.056	13.4	2.8
8	0.300	0.064	16.2	3.2
9	0.364	0.074	19.4	3.7
10	0.438	0.084	23.1	

Aus der Tabelle 2 können wir sehen, wie die Momentanunterschiedsschwellenwerte ΔE_s vom zweiten Wert 1.3 Lux $\cdot \sigma$ an, allmählich grösser werden, um an der neunten Schwelle den Wert 3.7 Lux $\cdot \sigma$ zu erreichen. Da, wie wir sahen, die Werte ΔE_s linear von den Werten ΔL_s abhängen und die letzteren Werte ihrerseits linear mit den L -Werten zusammenhängen, sind auch die Werte ΔE_s linear von den L - bzw. m -Werten abhängig. Dasselbe gilt natürlich auch in Betreff der Abhängigkeit der ΔE_s - und der E_s -Werte. Dagegen kann natürlich von einer Proportionalität

der ΔE_s - und der entsprechenden E_s -Werte, also von einer K o n s t a n z des Verhältnisses $\frac{\Delta E_s}{E_s}$, nicht gesprochen werden.

Die empirisch gezeigte Linearität der Momentanreizschwelle in ihrer Abhängigkeit von dem entsprechenden Grundmomentanreiz gilt wahrscheinlich nur bis zu Grundreizwerten, die nicht sehr weit von der absoluten Schwelle liegen, denn entsprechend wie es bei Dauerlichtreizen gezeigt worden ist, dürfte auch bei den Momentanreizen eine steiler als linear ansteigende Vergrößerung stattfinden. Es ist jedenfalls von Interesse, das einfach lineare Verhalten bei den niedrigsten Unterschiedsschwellen zu konstatieren.

Die in der Tab. 2 gegebenen ΔL_s -Werte sind inter- und extrapolatorisch mit Hilfe der Geraden der Abb. 2 erhalten, sie sind also empirische Werte. Die Werte der Momentanschwellen ΔE_s sind aus diesen Werten mit Hilfe der Gleichung $\Delta E_s = \frac{E_1}{2 \cdot L_1} \cdot \Delta L_s$ berechnet. Diese Gleichung ist auch empirisch fundiert und die ΔE_s -Werte vom Werte $s = 1$ an können demnach auch als empirisch angesehen werden. Die Einschränkung, dass die Werte nur von $s = 1$ an, also ausgenommen dem Wert $\Delta E_0 (= E_1 - E_0)$ als empirisch bezeichnet wurden, hängt damit zusammen, dass die Verwendung der obengenannten Gleichung zur Bestimmung der Werte ΔE_s nur vom Werte $s = 1$ an empirisch begründet ist, da die Gerade der Abb. 1 nur rechts von diesem Wert bekannt ist und ihre Verlängerung nach links eine Extrapolation ist, die, nebst der sich auf dieser Grundlage basierende Verwendung der genannten Gleichung, also nicht mehr als empirisch zu bezeichnen ist.

Wenn wir die Extrapolation erlauben und die Gleichung $\Delta E_s = \frac{E_1}{2 \cdot L_1} \cdot \Delta L_s$ also auch zur Berechnung der untersten Momentanunterschiedsschwelle verwenden, erhalten wir die glatt anwachsende, auch in der Tab. 2 angegebene Reihe der Momentanunterschiedsschwellen. Der unterste Wert (ΔE_0) wäre in dieser Weise berechnet $1.2 \text{ Lux} \cdot \sigma$, der folgende $1.3 \text{ Lux} \cdot \sigma$ usw. Der zweite Wert $\Delta E_1 = 1.3 \text{ Lux} \cdot \sigma$ und alle ihm folgenden sind rein empirische Werte, nur der erste Wert $\Delta E_0 = 1.2 \text{ Lux} \cdot \sigma$, gründet sich also auf eine nicht-empirische Extrapolation. Der empirische Wert von E_0 , welcher ja der Momentanreizwert bei Dunkelheit ist,

ist natürlich gleich Null, und nicht gleich $1.2 \text{ Lux} \cdot \sigma$, wie die Extrapolation der Abb. 1 angibt; diese beiden Werte sind in der entsprechenden Kolumne der Tab. 2 eingetragen. Und dem entsprechend ist, wie aus der Tab. 1 ersichtlich, der empirische Wert der untersten Momentanreizschwelle (früher mit E_1 bezeichnet) $\Delta E_0 = 2.4 \text{ Lux} \cdot \sigma$, also doppelt grösser als der extrapolatorisch bestimmte Wert.

Die eigenartige Diskontinuität in den empirisch fundierten Momentanreizunterschiedsschwellen, mit dem verhältnismässig grossen untersten Wert, ist schwer verständlich, besonders weil die Reihe der Dauerreizunterschiedsschwellen (ΔL_s) keine Diskontinuität aufweist, sondern vom niedrigsten Werte an, eine sich glatt und allmählich vergrössernde Serie bildet. Den Grund der genannten Diskontinuität möchten wir folgendermassen zu verstehen versuchen.

Die erste Dauerreizunterschiedsschwelle (ΔL_0) entspricht dem Unterschied zwischen dem Reizwert des ersten, des absoluten Dauerreizes (L_1) und der Dunkelheit, der Dauerdunkelheit (L_0). Und in der gleichen Weise entspricht die Dauerreizunterschiedsschwelle mit der Ordnungszahl s (ΔL_s) dem Unterschied der Dauerreize L_{s+1} und L_s . Sowohl in betreff der ersten Dauerreizunterschiedsschwelle wie in betreff der höheren entsprechenden Schwellen sind die Versuche auch so angeordnet gewesen, dass die zu vergleichenden Reize, um deren Unterschied es sich also handelt, Dauerreize waren. Insbesondere ist zu bemerken, dass auch der »Reiz« L_0 , also die Dunkelheit, im Verhältnis zu welchem der Reiz L_1 zu vergleichen ist, eine Dauerdunkelheit war.

Bei den Momentanreizunterschiedsschwellen sind die zu vergleichenden Reize aller höheren Ordnungszahlen solche von momentaner Dauer; bei jedem Reizunterschied ΔE_s sind also die Reize E_{s+1} und E_s , deren Unterschied ΔE_s ist, momentane Reize, Reize deren Zeitdauer kürzer als etwa 40σ ist. In betreff der ersten Momentanreizunterschiedsschwelle verhält sich die Sache aber anders. Die Bestimmung der absoluten Momentanreizunterschiedsschwelle (ΔE_0), welche identisch mit der absoluten Momentanreizschwelle (bezeichnet E_1) ist, geschah in einer Weise, bei der die Reize E_1 zwar von momentaner Dauer waren, die »Reize« E_0 , also die zwischen den Reizen geschalteten Dunkelheiten dagegen *nicht* momentan waren, sondern längere Zeit dauerten, also Dauerdunkelheiten waren. Gemäss der Ausführungsart der Versuche sind also die von uns empirisch erhal-

tenen, der untersten Ordnungszahl entsprechenden Werte ΔE_0 mit den, den höheren Ordnungszahlen entsprechenden Werten ΔE_s nicht ohne weiteres zu vergleichen. Ein sinnvoller Vergleich des untersten Momentanreizwertes mit den höheren entsprechenden Werten würde, nach dem Obenangeführten, also zur Bedingung haben, dass die Bestimmung auch des untersten Momentanreizes ein Vergleich des Momentanreizwertes E_1 mit dem Momentandunkelwert E_0 wäre. Diese Forderung läuft darauf hinaus, dass die Reizwerte Implikate der »entsprechenden« Empfindungswerte sind (siehe REENPÄÄ: »Über Wahrnehmen, Denken und messendes Versuchen«, 1947) und dass ein Vergleich verschiedener Reizwerte nur in dem Falle sinnvoll ist, dass die entsprechenden Empfindungswerte gleichartig sind. Wenn wir die Erlebniswerte mit kleinen, die implikativ entsprechenden Reizwerte mit grossen Buchstaben bezeichnen, können wir den Vergleich der untersten sowie der höheren Reizwerte einerseits bei den Dauer- und andererseits bei den Momentanempfindungen bzw. Reizen folgendermassen kurz andeuten.

Bei Dauerempfindungen bzw. -reizen:

Zu vergleichende Empfindungen l_{s+1} u. l_s sowie l_1 (absolute Dauerempfindung) u. l_0 (Dauerdunkelempfindung).

Implikativ entsprechende Reize L_{s+1} u. L_s sowie L_1 (absoluter Dauerreiz) u. L_0 (Dauerdunkelheit).

Bei Momentanempfindungen bzw. -reizen:

Zu vergleichende Empfindungen e_{s+1} u. e_s sowie e_1 (absolute Momentanempfindung) u. e_0 (Momentandunkelempfindung).

Implikativ entsprechende Reize E_{s+1} u. E_s sowie E_1 (absoluter Momentanreiz) u. E_0 (Momentandunkelheit).

Die Versuchsanordnung war also in allen anderen Hinsichten dieser Forderung gemäss, ausser inbetreff den Momentanempfindungen und -reizen der untersten Ordnungszahl, wo sie nicht dem obenstehenden Schema gemäss, sondern gemäss dem unterstehenden angeordnet war:

Zu vergleichende Empfindungen e_1 (absolute Momentanempfindung) u. l_1 (Dauerdunkelempfindung),

»entsprechende« Reize E_1 (absoluter Momentanreiz) u. L_0 (Dauerdunkelheit),

welche Anordnung nicht unserer Forderung entspricht.

Um die Versuche gemäss dem höherstehenden Schema anzuordnen, müssen bei der Bestimmung der absoluten Momentanschwelle die zwischen den Momentanreizen eingeschalteten Dun-

kelperioden also auch momentan sein, und wir machten demgemäss Versuche, bei denen der Versuchsperson kurzdauernde Lichtreize- und Dunkelpausen abwechselnd dargeboten wurden. Da die vorhin behandelten Versuche vor dem Kriege ausgeführt waren und die Versuchsapparatur seitdem abgebaut und wieder aufgebaut war, konnten nicht mehr neue Versuchsergebnisse erhalten werden, die ohne weiteres mit den alten Ergebnissen zu vergleichen wären. Da die frühere Versuchsperson uns auch nicht mehr zur Verfügung stand, wurde die Untersuchung der Bedeutung momentaner Dunkelpausen bei der Bestimmung der absoluten Momentanschwelle folgendermassen gestaltet. Der empirisch bestimmte Wert der Momentanschwelle bei Verwendung langdauernder Dunkelpausen ist, wie aus der Tabelle 2 ersichtlich, doppelt grösser ($2.4 \text{ Lux} \cdot \sigma$) als der theoretisch mit Hilfe der graphischen Geraden der Abb. 1 oder der entsprechenden Gleichung $\Delta E_s = \frac{E_1}{2L_1} \cdot \Delta L_s$ zu berechnende Wert ($1.2 \text{ Lux} \cdot \sigma$). Und

wir vermuteten, dass dieser theoretische Wert einem empirischen Werte entspräche, welcher mittels Verwendung momentaner Dunkelpausen zu erhalten wäre. Wenn dem so wäre, dann können wir am einfachsten mit Hilfe der Abb. 1 ersehen, dass dies bedeuten würde, dass der »Reizwert« einer Momentandunkelperiode (der Ordinatenwert E_0 in Abb. 1), welcher dem »Reizwert« Null einer Dauerdunkelperiode (der Abszissenwert Null der Abb. 1) entspricht, gleich $\frac{E_1}{2}$ ($=E_0$) ist, d. h. es würde besagen, dass die »Dunkelheit« einer Momentandunkelpause tiefer als diejenige einer Dauerdunkelperiode ist. Und da der Wert des Momentanreizes bei momentaner Dunkelperiode hiernach $\Delta E_0 = E_1 - E_0 = \frac{E_1}{2}$

ist, ersehen wir, dass er doppelt kleiner sein sollte als der entsprechende Wert (E_1), bestimmt bei Verwendung von Dauerdunkelperioden. Hiermit gründen wir auch unseren Versuch zur Prüfung der Gültigkeit dieser theoretischen Voraussage. An der Versuchsperson wurden zuerst absolute Schwellenreize mit momentanen Belichtungen und dazwischenliegenden Dauerpausen bestimmt und sogleich danach ähnliche Versuche, aber mit momentanen Dunkelpausen ausgeführt. Die Tabelle 3 zeigt das Ergebnis. Wenn die Dunkelpausen etwa 750σ dauern, erhalten wir für die unterste erste Reizschwelle den mittleren Wert

Tabelle 3.

Bestimmung der ersten Momentanreizschwelle mit einerseits zwischen die Momentanlichtreize eingeschalteten Dauerdunkelpausen (oberer Teil der Tabelle), andererseits Momentandunkelpausen (unterer Teil der Tabelle). Das Bild der Lichtfläche innerhalb der Fovea centralis.

Die Dauerdunkelpausen betragen 750 σ .

Dauer des Momentanlichtreizes t (σ)	Energie des Momentanlichtreizes E_1 (Lux $\cdot \sigma$)
20	0.570
40	0.628
80	0.751
100	0.935
Mittelwert 0.721 ± 0.122 Lux $\cdot \sigma$.	

Die Momentandunkelpausen betragen 60 bis 100 σ .

Dauer des Momentanlichtreizes t (σ)	Energie des Momentanlichtreizes ΔE_0 (Lux $\cdot \sigma$)
20 { Dauer der	0.233
40 { Dunkelpausen	0.426
3 \times t	
100 { Dauer der	0.488
{ Dunkelpausen = t	
Mittelwert 0.382 ± 0.0996 Lux $\cdot \sigma$.	

$E_1 = 0.721 \pm 0.122$ Lux $\cdot \sigma$, wenn dagegen die Dunkelpausen von der Dauer von nur 60 — 120 σ sind, ist der Mittelwert der ersten Reizschwelle $\Delta E_0 = 0.382 \pm 0.0996$ Lux $\cdot \sigma$, also ungefähr die Hälfte vom vorigen Wert, was quantitativ unserer theoretischen Auffassung entspricht. Bei dem Versuchen mit momentaner Dunkelpause flimmerte das Licht, wie zu fordern ist, da nur in diesem Fall die Versuchsperson zwischen Hell und Dunkel unterscheidet, und also einen Vergleich der Empfindungs- bzw. Reizwerte e_1 u. e_0 bzw. E_1 u. E_0 ausführt. Die Grösse des erhaltenen Wertes für E_1 ist bei dieser Versuchsperson kleiner als mit der anderen Versuchsperson. Das mag teilweise mit dem Adaptationszustand, teilweise mit einer individuellen Verschiedenheit zusammenhängen. Wir möchten in dem Ergebnis eine Stütze für diejenige Auffassung sehen, dass die Reizgrössen beim Vergleich miteinander, genau auf ihre Empfindungsimplikantien zu prüfen sind, also für die von uns auch vorhin betonte Auffassung von der Bedeutung der Begriffsanalyse der den phänomenalen Empfindungen »entsprechenden« Reizgrössen. Nur in dieser Weise können diese letztgenannten Grössen in einer richtigen Weise behandelt und verstanden werden.

Zusammenfassung.

1. Entsprechend der absoluten (der 1.) Schwellenempfindung (BLOCH u. a.), sowie der ihr nächsten überschwelligen Empfindungen von *Momentanlichtreizen* gelten Reizregeln von der bilinearen Form $L \cdot t = E$, wo L die Reizintensität, t die Reizzeitdauer und E die Energie des Reizes bedeuten.

2. Die den verschiedenen *momentanen* Empfindungsgrössen entsprechenden Reizenergiwerte E stehen in linearem Verhältnis zu den Reizintensitäten, welche bei *Dauerlichtreizen* mit den vorigen Empfindungsgrössen erlebnisäquivalent sind. Es gilt empirisch die Gleichung $E = (m + 1) \frac{E_1}{2}$, wo E_1 die Reizenergie der absoluten (der 1.) Momentanempfindungsschwelle ist und m eine Zahl bedeutet, die proportional der Reizintensität des Dauerlichtes ist.

3. Es wird gezeigt, dass in der *Nähe der absoluten Schwelle*, also bei den untersten Schwellenordnungszahlen, sowohl die *Dauer-* (ΔL_s) wie die *Momentanreizschwellen* (ΔE_s) eine lineare Abhängigkeit von den entsprechenden Dauer- (L_s) bzw. Momentangunderreizen (E_s) zeigen.

4. Bei den Dauerreizschwellen steigen die Werte der Reizschwellen (gemessen in Lux) von Schwelle zu Schwelle, vom ersten Werte an. Bei den Momentanreizschwellen gilt dasselbe Verhalten, aber nur von der zweiten Schwelle an. Die erste Momentanreizschwelle ist doppelt grösser als sie gemäss dieser linear sich vergrössernden Reihenordnung sein sollte (Tabelle 2). Es wird gezeigt, dass diese Diskrepanz darauf beruht, dass die hier gebrauchte und übliche Bestimmungsart der ersten Momentanlichtschwelle verschieden von der Bestimmungsart der Momentanlichtschwellen von höherer Ordnungszahl ist. In dem Falle, dass die Bestimmungsmethoden der begrifflichen Reizgrössen in allen Fällen auf gleichartigen, ihnen implikativ entsprechenden phänomenalen Empfindungsgrössen basieren, bekommt man eine vom ersten Momentanreizschwellenwert an glatt anwachsende Serie von Reizunterschiedsschwellen; die erste Momentanreizschwelle ist also bei dieser adäquaten Reizbestimmungsmethode die Hälfte kleiner als sie bei der üblichen Bestimmungsmethode ist.

5. Die empirische Bestätigung einer Konsequenz der theoretischen Auffassung des Zusammenhangs der begrifflichen Reiz-

größen und der phänomenalen Empfindungsgrößen zeigt, unseres Erachtens, die Bedeutung der von uns in anderem Zusammenhang versuchten Analyse des Reizbegriffes.

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Some Information on the Citric Acid Content of Bone Substance.

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Lund.

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In 1940 F. DICKENS published his surprising discovery that the bone substance of mice contains considerable amounts of Ci (= citric acid or citrate). The skeleton is, in fact, richer in Ci than the other organic systems of the mammal organism, containing the main part of the Ci-storage in the body. In the following year (1941) DICKENS published a more extensive report on these investigations. He stated that considerable amount of Ci had been found also in the bone substance of oxen ($> 0.272\%$), of puppies (1.31%) and of kittens (0.644%). At this time the high Ci-content of the bone substance of horses, sheep, swine, chickens, and a number of fish (pike, carp, herring, cod, and eel) had been determined in Thunberg's laboratory, Lund (THUNBERG 1941) and a short publication on these results was issued. It was thus fairly evident that Ci could be found in all bone substance.

Great practical significance can be attributed to DICKENS' discovery that bone-meal contains citric acid in a concentration of about 1.4% . The bone-meal was prepared commercially from coarsely pulverised bone, extracted with steam and finally dried and reduced to a fine powder. — The bone-meal in question seems to be identical to the Swedish pharmaceutical preparation *Calcii Phosphas crudus*, which preparation was entered in Ed. X of The Swedish Pharmacopæia in 1925 but excluded from Ed. XI in 1946. According to our investigation this Swedish preparation contained between 1 and 1.5% Ci. — No further information is given in Ed. X, however, concerning the origin and manufacture of the preparation.

It appeared from DICKENS' analysis that the Ci is not uniformly distributed throughout the bone substance. The concentration was especially high in the hard parts. DICKENS found, for example, a Ci-

content of 279 mg. per 100 gram in hard bone substance (of the anterior extremity of ox) but only 41 mg. in the red marrow from this bone.

NICOLAYSEN and NORBO (1943) have also contributed a number of values concerning the Ci-content of the bone system. They found a Ci-content of 0.50 % — 0.66 % in rats when the food was rich in Ca and P and contained vitamin D. If the food was poor in P but rich in Ca and free from vitamin D, the Ci-content was observed to be as low as 0.12 %—0.16 %. If the food contained vitamin D and was rich in Ca the Ci-content rose to 0.53 %—0.68 %. If the food was poor in Ca, the values sank to 0.42 %—0.52 %.

Values concerning the Ci-content in bone are reported also by CLASS and SMITH (1942). Determinations were made on composite samples of tibiae, fibulae, femura, humeri, radii, and ulnae from young mature male rats. A calculation of the average value of the individual values submitted by the American analysts gives the values 6.16 mg. of Ci per gram bone substance with a standard deviation of 0.46 mg. Ci.—The citric acid value of the entire skeleton would be 73.3 mg. (the average of 24 determinations) and the corresponding standard deviation 7.7 mg.

It has long been known that the metabolism of the bone system can be influenced by a large number of biological factors, as, for instance, age, sex, vitamins, hormones, etc. This is evidently valid also for the Ci-content. Prolonged administration of parathyroid hormone causes a substantial increase in the Ci of the bone, according to DICKENS. A kitten which was fed on a diet poor in Ca and showed signs of rickets, had a Ci-content of the skeleton of only 50 % of that of the control animal.

For half a century Ci has been thought to combine with Ca forming a compound which upon ionization yields a Ca-citrate complex. (See HASTINGS et al. 1934.) What is known (or assumed) about a Ca-citrate complex indicates, however, a relatively great solubility of this complex. The substance, on the other hand, in which Ci occurs in the skeleton, appears to be very difficult to dissolve. It seems as if the citrate in bone-meal is only slightly water-soluble. The insolubility of the Ci of the bone substance has also been observed in many experiments made in Lund concerning the possibility of basing the analysis of the Ci of the bone substance on the enzymatic method recommended by THUNBERG. This method has otherwise been very useful in analysis of blood and animal fluids on the whole.

One or two observations by KUYPER (1938) are elucidating in this connection. We must apparently reckon with complex compounds, containing not only Ca but also phosphate.

Thus, if Ci in a water solution is made to react with calcium and phosphate, and if these three substances are present in sufficient amounts and in suitable proportions, citric acid is quantitatively precipitated. Practically complete precipitation was also obtained from a solution containing 0.13 mg. of Ci per 100 ml. water. When no phosphate was present, practically no citrate was precipitated. The fact that

citrate is precipitated only when the calcium is present in excess of the amount necessary to react with phosphate and citrate, seems to indicate that it forms an insoluble calcium complex which is readily dissociated. (See also KUYPER 1945.)

It appears from this historical outline that the existing material concerning the Ci-content of the bone system is both scarce and inhomogeneous. The point is to make it more extensive and certain. It might be said about DICKENS' discovery, however, that it is of greater significance than the attention it has received (SENDROY 1945).

Method.

The citric acid determinations in this investigation have been performed according to the method of PUCHER-SHERMAN-VICKERY (1936). A few modifications have been introduced partly on account of recent experience with this method and partly due to difficulties in obtaining the necessary reagents. Pyridine was substituted by acetone or by a mixture of glycerin and water; ferro-sulfate by hydrogen peroxide, etc. — Towards the end the modification proposed by HUNTER and LELOIR (1945) was adopted in which pyridine was substituted by ethylene glycol and the decolorization of the excess potassium permanganate was performed with sodium nitrite. These changes made in the method during the period of the investigation have probably affected the significance of the analyses. In order to counteract this a number of control analyses have been carried out.

The bone material of man was obtained from autopsies at the Pathological Institute, Lund (Professor C. G. Ahlström). The animal material has been as fresh as possible. The values obtained did not seem to be influenced by the length of the storage period.

The skeletal parts used were freed by means of careful dissection from fat, connective tissue, periosteum and tendons, then dried preliminarily, finely divided, and, finally, dried and pulverized. Some helpful hints for working with bone substance can be obtained from LANG and HASLHAFFER (1935) and MICHAELIS (1930). — Naturally the material investigated was only macroscopically homogeneous. No microscopic preparations have been made.

The fact that the bone substance has two components, one spongy element (*Substantia spongiosa*) and one compact element (*Substantia compacta*) would seem to indicate the desirability of analyzing both these substances. It soon became evident, however, that an analysis of the compact substance rendered more information than analysis of the spongy part. The *Substantia compacta* has a uniform, homogeneous character. The *Substantia spongiosa*, on the other hand, varies in porosity and thus also in the amount of red marrow substance filling the cavities. DICKENS has already pointed out that the red marrow is very poor in Ci. The spongy bone substance will thus show all degrees between Ci-rich solid bone substance and Ci-poor marrow. The characteristic qualities of the bone tissue here seem to

evade evaluation. Quite a number of Ci-analysis have been carried out, however, confirming the opinions just given. The values varied between 0.5 % and 1.0 %.

The water and fat content of the bone substance is widely variable. Bone substance which had been freed from fat and water, on the other hand, showed a more homogeneous composition. The Ci-values obtained have therefore been stated in relation to the initial material free from fat and water (Cf. HUGGINS 1937). The values of such a water-free substance will naturally be comparatively higher than when related to a material with the original content of water and fat.

The Ci-values of fresh bone substance ($= X$), are easily calculated from the Ci-values of the dried bone substance ($= Ci_{sicc.}$) as well as the values of bone substance remaining after drying ($= R$) expressed in percentage of the initial amount used. The Ci-content of a certain amount of fresh bone substance is found quantitatively in the dried residue of the original material. The following equation is used.

$$100 \cdot X = Ci_{sicc.} \cdot R.$$

The Ci-content of the Bone Substance of Humans.

The following bone material of humans has been analysed: thigh, collar bone, rib, and vertebra.

In the table below the Ci-values are given which have been obtained for our human bone material. No individual values are reported but only the average values and the average errors of the isolated observations. (I am indebted to Amanuens Johannes-son for the statistical calculations.)

Table.

Average Ci-content of the thigh, collar bone, rib, and vertebra of man.

	Number of analyses	Average value in %	Average error of the observa- tion in %
<i>Femur, female</i>	7	1.62	0.24
<i>Collar bone, total</i>	39	1.88	0.27
<i>male</i>	19	1.87	0.24
<i>female</i>	20	1.89	0.31
<i>Rib, total</i>	32	1.75	0.33
<i>male</i>	17	1.80	0.32
<i>female</i>	15	1.70	0.32
<i>Vertebra, total</i>	32	0.89	0.32
<i>male</i>	18	0.71	0.17
<i>female</i>	14	1.11	0.34

It appears from the table that thigh, collar bone, and rib form a special group with an average Ci-content of 1.62 %, 1.88 %, and 1.75 % respectively. Arranged in the order collar bone, rib, and thigh, these three kinds of bone material give a number of equidistant average values with a difference of 0.13 %. The vertebra forms a group by itself with a Ci-value differing from the corresponding value for rib with no less than 0.86 %. — The reason why rib has been chosen for the comparison is that this bone represents both the sexes and shows the least difference from vertebra. This means that the Ci-value of collar bone shows a still more distinct difference from that of vertebra, if possible.

It can now be calculated from the present material with how great probability a difference obtained between the values of two different substances is due to an actual difference or is just a coincidence. The quotient of the difference and its average error is calculated. The degree of probability is obtained from specially constructed tables, under consideration of the number of degrees of freedom corresponding to the average error.

When treated in this manner the numerical material shows no significant difference in the Ci-values of bone substance from collar bone, rib, and thigh. Concerning the difference obtained between vertebra on one side and the remaining three kinds of bone substance on the other, however, these are so great that the probability of their depending on coincidence and having no real foundation is less than 1 : 1,000. This is valid both for the above-mentioned difference of 0.86 % between the values of vertebra and rib and for other relevant differences.

Concerning the difference between rib and vertebra from females, the differential value obtained is only 0.40 %, but the difference is statistically significant in this case, too.

The answer to the question whether there is any sexual difference in this material is that the vertebra seems to denote a sexual difference of 0.40 %, which only with a probability of less than 1 to 1,000 can be regarded as accidental. Vertebra of females appear thus to hold more Ci than that of males. The rather unfavourable conditions under which these investigations have been carried out, however, makes it desirable that they should be controlled. It is also possible that the lower Ci-value found in males is due to a larger amount of spongy substance.

It might be of interest to compare our results as to Ci, to previous results concerning the main inorganic components. Such

comparisons exist for different bones in the same individual. HOWLAND, MARRIOTT and KRAMER (1926) noted no difference in this respect, and BOGART and HASTINGS (1931—1932) observed no material difference in the inorganic composition of the inner and outer portions of bone. (SCHMIDT, C. L. A. and D. M. GREENBERG 1935.)

Sexual variation in the composition of bone have, however, been previously observed with a view to ash, Ca, and P percentage. Female rats contain a larger percentage of ash, Ca, and P between weaning and adult life than male rats. The bones of female rats are smaller (contain less organic matter and water) than males of the same age reared on comparable diet, but in spite of this they have at all ages a higher percentage than males of ash, calcium, and phosphorus. (Cf. References by HUGGINS (1937) and GARDNER and PFEIFFER (1943).)

Ci-values of Bird and Fish.

It was more by chance than due to biological motives that we chose the black-headed gull (*Larus ridibundus*) as a representative for birds in the investigation of the Ci-content of the bone substance in this group of vertebrates. The results in percentage are summarized in the following table:

M denotes average value, *s* the standard deviation.

Table.

	Pelvis	Shoulder blade	Thigh	Breast bone
M	2.08	2.63	1.20	2.67
s	0.18	0.33	0.30	0.44
	Humerus	Parietal bone	Coracoid bone	Vertebra of the neck
M	0.60	1.75	1.57	1.78
s	0.06	0.04	0.35	0.11

The average value and standard deviation are based on only 3 values which reduces their significance. The low humerus value is surprising.

The spine of the herring (*Clupea harengus*) was examined. The very high value of citric acid (5.25 %) in the first species examined led to 9 more Ci-analyses being carried out on different species with varying results. The average value of the determina-

tions was 4.27 % with a standard deviation of 1.21. The species analysed were rather varied in size, among other respects. The sex was not noted. — A few other species of fish were also examined with a view to the Ci-content of the spine. Mackerel (*Scombrus*) gave a Ci-value of 1.60 (the average value of 3 determinations), and cod (*Gadus callarias*) 2.01 % (the average value of 2 determinations).

Finally a number of analyses were carried out on the frog, type and sex unspecified. The frogs had been caught in the summer 1946 and were in rather poor condition at the time of the investigation (summer 1947), but the results were none the less amazing. The average value of the citric acid content of the bone substance was only 0.29 %, which value is many times lower than that usually found for other animals. — Several questions arise. To what extent is the low Ci-value of these frogs connected with the rôle of the skeleton in Ci-storage? Does the singular water economy of the frog play a part here? (OVERTON.)

Discussion.

The analyses reported above show beyond all doubt the general occurrence of Ci in considerable amounts in the bone substance of vertebrates. They will, too, help in creating a more correct and precise conception of the magnitude of the Ci-content. Ci probably occurs chiefly in the compact part of the bone substance. The richer the bone material is in "Substantia compacta" the higher the Ci-content under similar conditions; the presence of spongy substance, however, decreases the value. The difficulty in separating the two parts preparatively creates a corresponding difficulty in determining their Ci-content. — The main part of the inorganic bone substance occurs in sub-microcrystalline form (Cf. HUGGINS p. 120). Nothing definite is known as to the distribution in the crystalline and the liquid phases (the mother liquor) of the citric acid component of the bone substance. The Ci-content of the bone substance appears partly in a soluble form and partly in an insoluble form. This is confirmed by the experiments to extract finely divided bone-meal with water: part of the Ci is immediately dissolved but further dissolutions occurs very slowly. If an acid fluid is used for the extraction, for example 5 % trichloroacetic acid, the extraction, however, is rapid and easy. The

most natural explanation is that the citric acid occurs in a weakly dissociated state extremely difficult to dissolve in water, that is to say in the form which KUYPER has observed to be precipitated when mixing Ca, phosphate, and citrate under certain conditions.

One has long abandoned the view of the inorganic part of the bone system being a mixture of simple salts — Ca phosphate and Ca carbonate. The precipitation being dependent upon the degree of acidity does not support this theory. The salts of the skeleton are now assumed to occur in a complex combination (apatite?), but it is uncertain what kind of apatite: a hydroxyl-apatite or carbonate-apatite or a combination of both forms. The occurrence and conditions of Ci in the bone substance do not disagree with the apatite-theory.

It should be observed that the discovery of citrate in the bone substance complicates matters.

Even the amount in which Ci occurs indicates its positive significance. It is true that the possibility of further components in the bone substance should be taken into account, especially blood components in weak concentration, the result of an equalizing diffusion. Concerning the citric acid of the skeleton, however, the situation is probably more complex. It seems most likely that the difference in concentration is due to a precipitation within the bone substance in the form of a complex compound.

The fact that so large a quantity of a familiar substance has hitherto escaped detection in bone analysis is surprising as has also been pointed out by DICKENS. He draws attention to the fact, however, that it is upon bone ash that the most accurate and complete analyses have been made. In these the citrate would presumably appear as an addition to the carbonate fraction. In ash analysis of bone we should thus reckon with a correction factor corresponding to the amount of carbonate formed from citrate.

There is still another thing that should be taken into account in bone analyses. For a long time the relation between basic and acid equivalents of bone wet ash has been an object of discussion. (Cf. HUGGINS p. 127.) This discussion should be continued while considering new subsequent factors. HUGGINS finishes his report on the problem in 1937 with the following words: "Further complete acid-base studies are needed on this important point."

Summary.

The author summarizes our scant knowledge up to the present time of the citrate content of the bone system and reports new determinations carried out by him on humans, birds (*Larus ridibundus*), fish (*Clupea harengus*, *Gadus callarius* and *Scomber scombrus*). The citrate determinations have been made by means of the colorimetric variation of Stahrés pentabromacetone method introduced by PUCHER, SHERMAN and VICKERY, and finally with the modification worked out by HUNTER and LOLOIR. The amount of citrate found has been put in relation to water-free (ev. also fat-free) substance. When expressed in this manner the value for humans, birds, and fish vary between one and a few percent. — The Ci-value in the frog, kept during the winter and not used until late in the summer, was about 0.3 %.

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Depressor and Pressor Activity of Extracts from the Aortic Wall of Cattle.

By

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In 1934 BACQ suggested that adrenaline was the chemical mediator of sympathetic inhibitory impulses whereas nor-adrenaline caused the excitatory response. This assumption actualized the early observation by BARGER and DALE (1910) that nor-adrenaline mimics some of the effects of sympathetic stimuli better than does adrenaline. In 1937 CANNON and ROSENBLUETH gave a review of the experimental evidence which led them to postulate that adrenergic nerve action involves the liberation of a common mediator, adrenaline, which in the reacting cells forms two different substances, called sympathin E and sympathin I, producing excitatory and inhibitory actions respectively. In 1942 BLASCHKO gave chemical evidence for the formation of nor-adrenaline in the body.

The works of VON EULER (1945, 1946) have thrown new light on this problem. He found that extracts from various organs including adrenergic nerves exerted effects which were, after removal of contaminating factors with Fuller's earth, identical with those of nor-adrenaline and corresponded to the postulated sympathin E. These results have recently been confirmed by BACQ and FISCHER (1947) and are also in full accordance with the findings of GADDUM and GOODWIN (1947).

On the cat's blood pressure, the crude organ extracts, made according to EULER, exert a double action. First there is an initial fall in blood pressure which is then followed by a marked rise. This depressor activity can be removed by treating the extracts with Fuller's earth at a slightly acid reaction. After this treatment

the extracts as a rule give a pure rise in blood pressure. With this method it has also been shown that blood contains a sympathomimetic substance with the actions of nor-adrenaline (EULER and SCHMITERLÖW, 1947).

The present investigation, which deals with the depressor and pressor action of extracts from the aortic wall of cattle, is a first attempt to a closer investigation of the rôle played by the blood vessels in producing vaso-active substances.

Methods.

The method of extraction from aortic wall was that described by EULER (1946).

The aortic arch and the abdominal aorta were collected from newly killed cows. The vessels were as soon as possible freed from connective tissue, grinded and extracted for two hours with acid alcohol. After filtration on a Buchner funnel the filtrate was evaporated in vacuo to a small volume and shaken with ether in order to remove the lipids. The ether was then removed and the volume of the extract was made to correspond with the weight of the minced material so that 1 ml of the extract corresponded to 2 g of fresh material. The pH of the extract was throughout these procedures kept on approximately 4.5.

These extracts, in the following called aortic extracts, were tested on the blood pressure and the non-pregnant uterus in situ of the cat in chloralose. The effect of the extract was compared with that of adrenaline (PARK, DAVIS & Co) and of dl-nor-adrenaline (arterenol). Sometimes it was necessary to sensitize the cat towards these sympathomimetic agents as well as towards the extracts by giving 8 mg/kg cocaine hydrochloride i.m. Dihydroergotamine¹ 0.5 mg/kg i.v. was used to inhibit or reverse the pressor action of adrenaline.

As has been mentioned, extracts of this kind give an initial lowering of the blood pressure and this action can be removed by repeated treatment with Fuller's earth at about pH 4. In order to study the nature of this depressant substance, the Fuller's earth, after having been used for adsorption, was treated with an aqueous 10 % pyridine solution at pH 8 for half an hour under occasional stirring. After filtration the pyridine was removed in vacuo and the pH adjusted to neutral. These extracts, in the following called Fuller-extracts, were then tested as will be described later.

Results.

1. The Pressor Action (Aortic Extracts).

A. Cat's Blood Pressure.

The aortic extracts cause a marked initial fall in blood pressure which is sometimes followed by a marked elevation. This is,

¹ Kindly put at my disposal by professor E. Rothlin, Sandoz A. G., Basel.

however, not always the case, obviously due to the existing blood pressure level. When this is high the predominant effect of the extracts is a pure fall in blood pressure, when low there is usually a secondary rise. After treatment once with Fuller's earth the depressant action is diminished and a secondary rise is observed. After a second treatment the depressant phase has as a rule disappeared and there is now only a marked elevation of the blood pressure (fig. 1).

After treatment with iodine at pH 8 this depressor activity is reduced almost to annulment.

When a pure adrenaline solution or a solution of nor-adrenaline is treated twice with Fuller's earth a slight decrease in activity is noticed. When testing the amount of pressor substance obtained per unit of weight from the aortic wall it thus seems necessary to test it against Fuller's earth-treated adrenaline. Using this method of testing, the aortic walls were found to contain an amount of pressor substance, equivalent to 2 μ g adrenaline per gram.

After a dose of 0.5 mg dihydro-ergotamine per kg of body weight there is usually a reversal of the adrenaline effect but only a moderate decrease in the pressor effect of nor-adrenaline. The pressor action of the aortic extracts are usually not reversed by ergotamine, which indicates that the active substance corresponds to nor-adrenaline (fig. 2). Sometimes, however, an extract may in one cat show this nor-adrenaline-like action but in another cat its pressor action is reversed by ergotamine, indicating the presence of adrenaline (fig. 3).

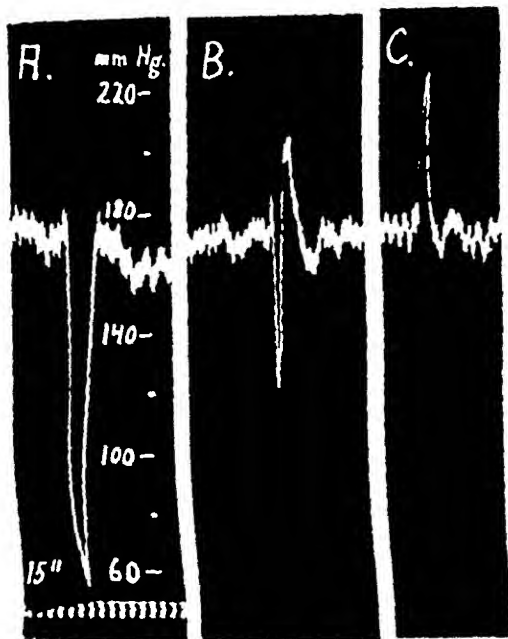


Fig. 1. Blood pressure, cat, chloralose.

- A. Untreated aortic extract, corresponding to 1 g aortic wall.
- B. The same extract treated once with Fuller's earth.
- C. The same extract treated twice with Fuller's earth.

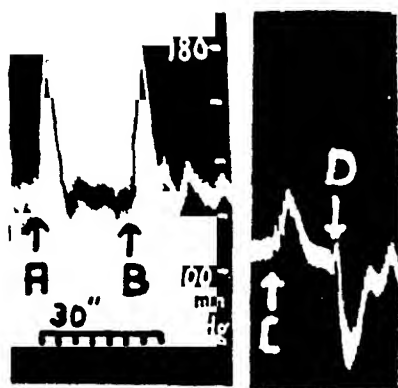


Fig. 2. Blood pressure, cat, chloralose.
 A. $2 \mu\text{g}$ adrenaline, treated twice with Fuller's earth.
 B. Aortic extract, corresponding to 1 g aortic wall, treated twice with Fuller's earth.
 Between B and C 0.5 mg/kg dihydro-ergotamine i.v.
 C. The same as B.
 D. The same as A.

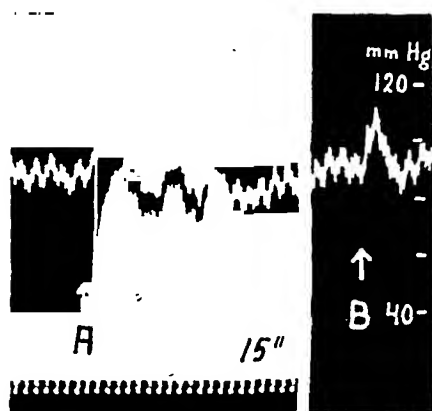


Fig. 3. Blood pressure, cat, chloralose.
 After 0.5 mg/kg dihydro-ergotamine i.v.
 A. Aortic extract, corresponding to 1 g aortic wall, treated twice with Fuller's earth.
 B. $1 \mu\text{g}$ nor-adrenaline.

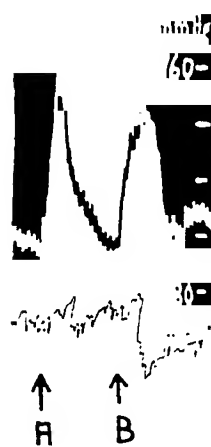


Fig. 4. Cat, chloralose, cocaine-HCl 8 mg/kg i.m.
 Upper curve: blood pressure.
 Lower curve: non-pregnant uterus.
 A. Aortic extract, corresponding to 1 g aortic wall, treated twice with Fuller's earth.
 B. $2 \mu\text{g}$ adrenaline.

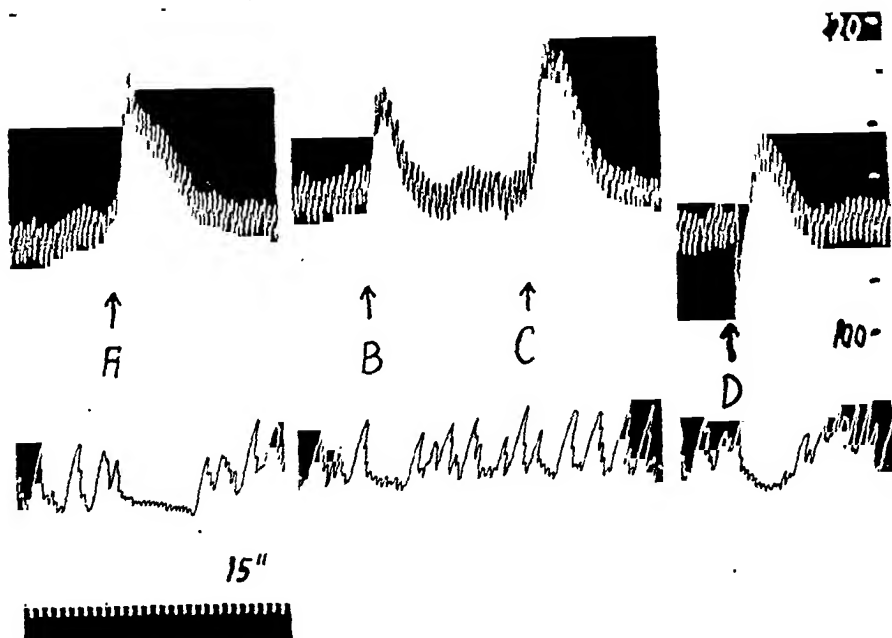


Fig. 5. Cat, chloralose.
Upper curve: blood pressure.
Lower curve: non-pregnant uterus.

- A. 4 μ g adrenaline.
- B. Aortic extract, corresponding to 3 g aortic wall, treated twice with Fuller's earth.
- C. 4 μ g nor-adrenaline.
- D. Fuller-extract, corresponding to 4 g aortic wall.

B. Cat's Non-pregnant Uterus in Situ.

On the non-pregnant uterus of the cat adrenaline causes a relaxation whereas nor-adrenaline does not exert any visible changes in tone. When the depressor activity had been removed from the aortic extracts by treating them with Fuller's earth, these extracts have as a rule no effect on the uterus (fig. 4). It sometimes happens, however, that the aortic extracts with pure pressor effect give a slight relaxation of the uterus, thus more corresponding to adrenaline than to nor-adrenaline (fig. 5). If the pressor effect, however, is preceded only by a slight depressor phase the uterus shows a distinct relaxation (fig. 6).

2. The Depressor Action (Fuller-extracts)

A. Cat's Blood Pressure and Non-pregnant Uterus in Situ.

The extracts from the material absorbed by the Fuller's earth give a sharp fall in blood pressure, usually followed by a secondary

rise. This effect on the blood pressure is accompanied by a relaxation of the uterus during the secondary increase in blood pressure (fig. 5).

The action on the blood pressure still exists after boiling the Fuller-extracts in normal hydrochloric acid for 10 minutes.

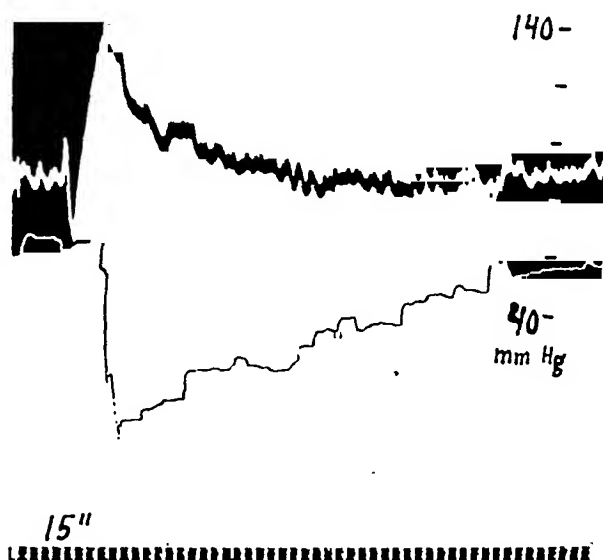


Fig. 6. Cat, chloralose.
Upper curve: blood pressure.
Lower curve: non-pregnant uterus.

Aortic extract, corresponding to 1 g aortic wall, treated once with Fuller's earth.

The sharp fall in blood pressure, accompanied by a relaxation of the uterus is very similar to the effect of histamine. This correlation was further strengthened by the finding that a previous intravenous dose of 10—20 mg of dimethylaminoethylbenzyl-aniline,¹ the antihistaminic substance described by HALPERN (1942), greatly reduced these effects of the extracts and to the same extent as histamine was found to be counteracted.

B. Isolated Small Intestine of Guinea-pig.

The Fuller-extracts contract the isolated small intestine of the guinea-pig. The amount of this contracting substance in these ex-

¹ "Lergitin", Recip, Stockholm.

tracts corresponds to about 1 μ g of histamine dihydrochloride per gram fresh aortic wall. The contraction of the intestine is quite abolished by previous addition to the suspension fluid of 2 μ g of the above-mentioned antihistaminic substance (fig. 7). This substance seems to be rather specific in its action against histamine, the action of acetylcholine and choline only being insignificantly influenced.

C. Action on the Human Skin.

When applying a drop of the Fuller-extract on the skin of the fore-arm and then making a pin-prick in the skin under this drop there soon develops the characteristic response, designated by LEWIS as "triple response". The appearance and duration of the wheal reaction closely corresponds to the action of a histamine solution with equal strength as determined by its intestine contracting power.

D. Diazo Reaction.

The Fuller-extracts show a positive Pauly's diazo reaction. Probably due to impurities which destroy the diazonium salt, this reaction, however, becomes positive only when using reagents which are about 5—10 times the strength of those originally suggested by PAULY.



Fig. 7. Isolated small intestine of guinea-pig. Bath-volume 20 ml.

- A. Fuller-extract, corresponding to 1g of aortic wall.
- B. 2 μ g of antihistaminic substance.
- C. The same as A.

Discussion.

EULER (1945, 1946) found that the pressor activity in extracts from various organs could be attributed to the presence of a substance which in its action closely corresponds to nor-adrenaline.

The pressor activity in the aortic wall extracts, made according to Euler's method, also seems to correspond to nor-adrenaline, but for two reasons. Sometimes it occurs that the pure rise in blood pressure is accompanied by a slight relaxation of the uterus and sometimes it occurs that the pressor effect of the extracts are reversed after ergotaminization. These two facts could be taken as an evidence that the pressor activity in some cases is due to adrenaline. When the depressor phase is not quite removed the

relaxation of the uterus is more pronounced. It seems obvious from the present investigation that these rather puzzling facts must be considered in relation to the nature of the depressor substance.

The depressor activity can be removed by treating the extracts with Fuller's earth. When this depressor substance (or substances) has been adsorbed to the Fuller's earth it has been eluted from this and tested separately. It then became obvious that the depressor activity at least to a great extent is due to the presence of histamine. The reasons for this statement are due to the following facts: 1. On the cat these Fuller-extracts give a marked fall in blood pressure usually followed by a secondary rise. This effect can be considerably diminished by pre-treatment of the cat with an antihistaminic substance. 2. On the isolated small intestine of the guinea-pig these extracts cause a strong contraction which is abolished by a very small dose of antihistamine. 3. They give a typical histamine wheal reaction on the skin. 4. They show a positive diazo reaction.

In the light of the fact that the aortic extracts contain rather large amounts of histamine (approximately 1 μ g per gram of fresh tissue) it becomes clear that when not all of the histamine is removed the secondary rise in blood pressure can be accompanied by a relaxation of the uterus, since it is well known that histamine, especially when the blood pressure level is low, causes an increased output of adrenaline from the adrenals (DALE, 1920, KELLAWAY and COWELL, 1922, HOGBEN, SCHLAPP and MACDONALD, 1924, BURN and DALE, 1926, FELDBERG, 1929). Then to the pressor action of the extract is added the action of adrenaline, produced by the test-animal itself. Even when all the depressor activity is removed, as judged from a pure rise in blood pressure, there may still be some traces of histamine left, the depressor activity of which is masked by the pressor substance, but which nevertheless may cause an output of adrenaline, sufficient to give a relaxation of the uterus.

The same argument holds true also for the apparently adrenaline-like action after ergotamine. In those cases where the pressor action of the aortic extracts is reversed by ergotamine we might suppose that this is due to a small amount of histamine which causes an output of adrenaline, masking the effect of the extract's pressor substance, the action of which is in itself already diminished by the ergotamine.

The cases where the extracts exert an adrenaline-like action are few, but they still nevertheless exist. This might possibly be due to a high susceptibility towards histamine in some of the cats.

After treating the extracts with iodine in alkaline solution the pressor activity is destroyed and one might then suppose that any histamine remaining after the treatment with Fuller's earth would then come into visible action. But unfortunately, histamine is also destroyed by iodine in alkaline solution.

Summary.

The depressor and pressor activity of extracts from the aortic walls of cattle have been investigated, using the extraction method described by EULER.

The depressor action seems to a great extent to be due to the presence of histamine in the extracts.

The pressor action of the extracts is due to some substance which in most cases mimics the action of nor-adrenaline but in some cases is more like adrenaline. This difference in the action of the extracts is not due to the presence of different pressor substances but to the presence of contaminating histamine. Traces of histamine, though masked in its action on the blood pressure by the pressor substance, may cause a liberation of adrenaline in the test-animal itself.

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Blood Lactate and Oxygen Debt After Exhaustive Work at Different Oxygen Tensions.

By

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On the International High Altitude Expedition to Chile EDWARDS (1936) made the unexpected observation that when one worked at the limit of his strength the blood lactate concentration was lower than it had been at maximal exertion at sea level. This effect became more pronounced with increasing altitude, and at 5,300 m the blood lactate after exhaustive work was hardly above the usual resting value. The diminished accumulation of lactic acid was assumed by EDWARDS to be due to a protective mechanism. DILL (1938) also regards this phenomenon as caused by useful safeguards against overexertion and in discussing EDWARDS' findings writes: "It is as though the body, realizing the delicacy of its situation with regard to oxygen supply, sets up an automatic control over anaerobic work which renders impossible the severe acid-base disturbances which can be voluntarily induced at sea level".

HILL, LONG and LUPTON (1924) in studying the effect of breathing air mixtures rich in oxygen on the capacity for work found a corresponding paradoxical relationship between oxygen tension and maximum oxygen debt, the latter being considerably larger if the oxygen tension in the inspired air is higher than normal. HILL, LONG and LUPTON had not expected this, but rather the reverse, and tended to regard it as being connected with the diminished distress associated with the increased oxygen content of the arterial blood.

In the present paper we have studied the effect of acute anoxia on the capacity for work, and on the ability to accumulate lactic

acid and to contract an oxygen debt. In such experiments there are no disturbances of the acid-base balance of the blood previous to the work in contradistinction to experiments made on subjects acclimatized to high altitude. In some cases the alkaline reserve of the subjects was lowered previous to the experiments by ingestion of large amounts of NH_4Cl . Furthermore we have repeated the experiments of HILL, LONG and LUPTON on subjects breathing 100 per cent oxygen.

Methods and Procedures.

The capacity for work was determined in two ways, viz. 1) as the maximum work that could be performed at a given rate (2,000 mkg per minute) and 2) as the shortest possible time necessary for performing a given amount of work (9,860 mkg).

The experiments were performed on a KROGH's bicycle ergometer, which besides the normal electrical brake was furnished with a frictional braking device (ASMUSSEN and BØJE 1945) to be used in the second series of experiments.

The blood lactate was estimated on capillary blood from the finger according to EDWARDS (1938). The highest value, which is reached 3 to 6 minutes after cessation of work, was used. The maximal oxygen uptake during work and the oxygen debt (extra oxygen uptake for the first 25 minutes after cessation of work) were determined by the Douglas-bag method. The anoxia was produced by having the subjects breathe a mixture of nitrogen and oxygen (12 per cent) from a large rubber bag being continuously refilled from a cylinder. Pure oxygen was administered in the same way. The alkaline reserve was determined as the total CO_2 content of the arterialized capillary blood on the VAN SLYKE apparatus.

As subjects served 3 young well trained students. All experiments were made in the morning under standard conditions.

Results.

Table 1 shows the results from the experiments on subject P. Th. and subject C. P. working at a constant rate till exhaustion. In both subjects the total work performed is greatly increased with increasing percentage of oxygen in the inspired air. The maximal oxygen consumption during work is considerably lower in 12 per cent oxygen than in normal air. In both subjects the oxygen debt seems on an average to be slightly higher in low oxygen than in normal air, whereas the lactic acid concentration tends to be a little lower in 12 per cent oxygen than in normal air and in pure oxygen.

Table 1.

Work at Constant Rate: 2,000 mkg/min.

Subject	Inspired air	Duration of work min.	Total work mkg	Max. O ₂ consump. during work l/min	O ₂ -debt in 25 min. recovery l	Max. blood lactate mg%
P. Th.	12 % O ₂	2.05	4,100	2.26	8.29	117
	"	1.88	3,770	2.48	8.37	94
	21 % O ₂	3.35	6,700	3.70	8.85	135
	"	2.97	5,940	3.11	7.15	110
	100 % O ₂	4.10	8,200	—	—	109
	"	4.92	9,840	—	—	128
C. P.	12 % O ₂	2.42	4,840	2.61	10.86	108
	"	2.42	4,840	2.32	11.71	110
	21 % O ₂	3.29	6,570	3.18	9.61	118
	"	3.62	7,240	3.53	11.63	136
	100 % O ₂	4.14	8,270	—	—	114

Table 2.

*Total Work 9,860 mkg.
Subject P.L.*

Inspired air	Date	Performance time min.	Max. O ₂ -consumpt. during work l/min.	O ₂ -debt in 25 min. recovery l	Max. blood lactate mg %
12 % O ₂	3/28	6.27	2.02	14.2	212
"	4/4	7.62	2.37	10.9	175
21 % O ₂	4/2	5.01	3.89	12.4	140
"	4/9	5.54	3.81	11.3	126
100 % O ₂	4/11	5.01	—	—	112
"	4/16	4.89	—	—	134

Table 2 shows the results from the experiments on P. L. whose capacity for work was determined as the shortest possible time necessary for performing a work of 9,860 mkg. It is seen that the performance time is considerably increased in low oxygen and slightly decreased in pure oxygen as compared with the performance time in normal air. The maximal oxygen consumption during work when breathing 12 per cent oxygen is much lower than in the normal experiments. There seems to be no large difference between the oxygen debt in 12 per cent oxygen as com-

Table 3.
Experiments with Lowered Alkaline Reserve.

Subject	Date	Con- dition	Total ar- terial CO ₂ Vol %	Dura- tion of work min.	Total work mkg.	Max. O ₂ - uptake during work l/min.	O ₂ -debt in 25 min. recovery l	Max. blood lactate mg %
P. Th. (constant rate 2,000 mkg/min)	4/24	normal	58	3.32	6,640	3.41	8.76	117
	4/25	»	54	3.86	7,720	3.76	9.83	124
	4/29	NH ₄ Cl-	41.5	3.55	7,100	3.64	8.06	115
	4/30	acidosis	43.5	4.02	8,040	3.66	9.31	123
P. L. (total work 9,860 mkg)	4/25	normal	49	5.25	9,860	—	12.21	128
	4/30	NH ₄ Cl-	39.5	5.29	9,860	3.76	10.83	136
	5/2	acidosis	33	5.12	9,860	4.32	10.43	114

pared to the oxygen debt in normal air and the scattering of the single determinations allows no conclusions to be drawn as to eventual smaller differences. The maximum lactic acid concentration is about the same in pure oxygen and in normal air but is distinctly higher in 12 per cent oxygen.

In the experiments in table 3 the alkaline reserve was lowered by giving the subjects about 15 grams of NH₄Cl on the day previous to the experiment and about 5 g early in the morning of the experimental day. The experiments on P. Th. were performed at a constant rate of work till exhaustion and those on P. L. with a constant amount of work performed in the shortest possible time. It is seen from table 3 that the lowering of the alkaline reserve has had no appreciable effect on the capacity for work or on the maximum oxygen uptake during work. Nor were the oxygen debt or the maximum lactate concentration in the blood affected.

Discussion.

The present experiments have shown that by an acute lowering of the oxygen tension of the inspired air the capacity for maximal work is considerably reduced, whereas the ability to contract an oxygen debt and to accumulate lactic acid is practically unchanged with the exception of the experiments in table 2 in which the lactic acid is considerably higher in low oxygen than in normal air. The explanation for this exception may be that

the possibility for a diffusion equilibrium between the lactic acid content in the muscles and the blood in the actual working period is greater due to the longer duration of the work. The opposite tendency for the blood lactates in table 1 to be lower in low oxygen than in normal air may be explained correspondingly as being due to the shorter duration of the work in low oxygen in this type of exercise. It therefore seems natural to assume that the limit for maximum work is set at a critical value of the lactic acid concentration in the muscles, which is of the same height in low oxygen and in normal air. This assumption is supported by the experiments in pure oxygen, which show, that although the capacity for maximal work is considerably increased, the maximal lactic acid concentration is about the same as in normal air.

The results of the present experiments are at variance with the results of those of EDWARDS (1936) in which the ability to accumulate lactic acid was greatly decreased during a sojourn at high altitude. EDWARDS' subjects had spent a considerable time at high altitude and as suggested by DILL (1938) their failing ability to accumulate large amounts of lactic acid might be due to the lowered alkaline reserve existing under such conditions. In the experiments in table 3 the percentage decrease in alkaline reserve corresponds to the decrease in alkaline reserve found in subjects living at altitudes between 3.7 and 6.0 km as estimated from the graph given by DILL, TALBOTT and CONSOLAZIO (1937). But as shown in table 3 neither the capacity for work nor the ability to accumulate lactic acid were impaired by this lowering of the alkaline reserve.

Judging from these experiments it seems doubtful to us to consider the diminished ability for formation of lactic acid at high altitude as the effect of a useful acclimatization but rather as being due to a general weakening of the subjects living under the stresses of high altitude. Such a weakening could be assumed to lower the ability to perform anaerobic work and, therefore, to produce lactic acid, the effect being brought about by an impairment of the central nervous system which is known to be specially sensitive to low oxygen tensions. A weakening of the oxygen transporting system (generally or specifically of the heart or diaphragm as supposed by EDWARDS), but with the ability to perform anaerobic work remaining intact, could on the other hand not decrease the ability to accumulate lactic acid.

A decreased ability to perform anaerobic work might also be explained as being due to local changes in the muscles, if for instance the sojourn at high altitude caused a decrease of the content of glycogen in the muscles or an inhibition of the transformation of muscle glycogen to lactic acid.

Summary.

On the International High Expedition to Chile EDWARDS (1936) found that the maximum lactate concentration after exhaustive work was much lower than at sea level. In the present experiments with acute changes of the oxygen tension in the inspired air the following results were found.

1) At a decreased oxygen tension the capacity for work was lowered, but the maximum lactate concentration was, practically, the same or — in one type of work — higher than in normal air. Also the oxygen debt was practically the same as in normal air.

2) In pure oxygen the capacity for work was increased but the maximal blood lactate concentration was practically the same as in normal air.

3) A lowering of the alkaline reserve by ingestion of NH_4Cl corresponding to the decrease found at altitudes from 3.7 to 6.0 km had no effect on the capacity for work or on the maximum blood lactate concentration and oxygen debt after exhaustive work.

According to these results it seems doubtful that the failing ability to accumulate large amounts of lactic acid at high altitudes is the effect of a useful acclimatization, but that it is due rather to a progressive weakening of the subjects making them less able to perform anaerobic work.

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From the State Pharmaceutical Laboratory.

The Influence of Muscular Exercise on the Tolerance of Digitalis in Guinea-Pigs.

By

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Experience shows that heart patients, who have got a certain maintenance dose of digitalis in hospital, after leaving very soon get back symptoms of cardiac decompensation. We thought that this might be partly due to a change in the need of digitalis following on increased physical movement subsequent to being in hospital. We have not been able to discover any clinical or experimental investigations on animals in support of this. We have therefore taken up the question and have begun to investigate the influence of muscular exercise on the tolerance of digitalis in guinea-pigs.

Methods and Results.

a) Experiments with Muscular Exercise.

The experiments were carried out on guinea-pigs weighing 300—400 g. The animals were divided into 3 groups, the first and the second each comprising 20, and the third 15.

Groups I and II were given daily subcutaneous injections of digitalis (Digitotal Astra, 1 ml respond. 10 cg fol. dig. internat. stand.). When applied subcutaneously, LD 50 of this preparation was 0.25 ml per 100 g guinea-pig. The third group served as a control and was given no digitalis. Immediately after every digitalis injection the animals in group I were allowed to run about as long as they had the strength, though not longer than one hour,

while the ones in group II were not exercised. The exercise took place on a moving platform, which consisted of a box, about 2 m long, and on the bottom there was a mat which kept on moving at the rate of about 10 m per minute. The guinea-pigs were kept on the run, there being a rotating broom fixed at one of the short ends to prevent them from sitting still. Times and weights were followed. Before the tests with digitalis were begun, the animals were trained for a few days in running.

During the first test day all the animals received Digitotal in a dose of 0.125 ml per 100 g guinea-pig, *i. e.* $\frac{1}{2}$ of LD 50. Several of the animals in both groups were rather affected, being weak and dull, at the same time as they showed a reluctance to move. Most of the animals that were set to run could keep on for about 15—30 minutes. After a few hours the symptoms of the digitalis injections had disappeared. It was the same thing on the second test day, and in consequence the injections were afterwards divided up into 2 doses per day (2×0.075 ml per 100 g guinea-pig), the animals in group I now running for one hour twice a day. On the third test day the animals were less affected by the digitalis, and during the following days the immediate discomfort caused by the injections became less and less. The dose was therefore increased to 2×0.125 g per 100 g animal and day. This quantity of digitalis (half the lethal dose twice a day) was now given to the animals for three weeks. With this strong dosage the animals were almost as much affected during the first few days as they were during the first few test days. They were able to accomplish the running for 15—30 minutes; the second run was usually somewhat shorter than the first. After some days the animals stood up better to the dose and were able to increase the time from 30—45 minutes per time and per animal. Most of the guinea-pigs, however, still showed the effects during the first hours after the injections, though not to such a pronounced degree as during the first test days, which will also be seen from their running times. The following table gives the mortality during the test time.

Table 1.

	Group I (exercised)	Group II (not exercised)
Dead	2 ¹	11 ²
Survived	18	9

¹ Dead after 2 resp. 12 days.

² Dead after 10—25 days.

The result has been treated statistically according to FISHER (1936) with χ^2 analysis.

P denotes probability that different groups belong to the same population.

$P \equiv 0.05$ denotes that there is a probable difference between the groups.

$P \equiv 0.01$ denotes that the difference is very probable.

$P \equiv 0.001$ denotes that the difference is significant.

For the difference between groups I and II $P = 0.01-0.001$.

During the month that the test was going on the surviving animals had each received 5.3 ml digitotal per 100 g animal subcutaneously, corresponding to 0.53 g fol. dig. internat. stand. None of the animals died during the first few hours after the digitalis injections. All the guinea-pigs increased in weight during the test time with the exception of the ones that died, and they decreased in weight only during the last 2—3 days. If we exclude these days, there is no difference in the weight increase, either between the two groups that received digitalis or between these and the controls in group III.

The heart action was followed by electrocardiograms. The animals were fixed in recumbent positions and needle electrodes applied hypodermically in the right and left fore-legs and the left hind-leg. The customary 3 leads were registered, firstly before, secondly after the digitalis treatment and muscular exercise. The heart rate was usually about 300 per minute, though occasionally it could go down to about 250. The T-waves were upright or isoelectric. The atrioventricular conduction time was 0.06—0.08 seconds. There were no changes in the RST segment until the animals were in a really bad way or until just before they died. It was not possible to observe any continuous changes. Occasionally it was possible to observe some decrease in the heart rate, a depression of the T-wave and a slight deviation of the RST segment, but all of this was of a transitory nature. It was not possible to register any electrocardiographic difference between those animals which ran on the moving platform and the ones which did not exercise.

b) The Determination of the Minimum Lethal Dose of Digitalis on the surviving Guinea-Pigs according to Knafli-Lenz.

The smallest quantity of intravenously injected Digitotal, converted into mg fol. dig. intern. stand. that was necessary to

kill the animals was determined on the surviving guinea-pigs. Knaffl-Lenz' method was adopted with the modifications applied by the State Pharmaceutical Laboratory (KÄLLROT and RYDIN, 1943). The result will be seen from the following table.

Table 2.

Group I			
(digitalis-treated, exercised, 16 animals) . . .	16.0 ± 0.79	mg/100 g	guinea-pig
Group II			
(digitalis-treated, not exercised, 8 animals) . .	13.5 ± 0.36	,	,
Group III			
(non-treated controls, 15 animals)	17.8 ± 0.95	,	,

Two of the surviving guinea-pigs in group I and one animal in group II died under the urethane anesthetic before biological assays had been started.

The T-analysis according to FISHER shows that the difference between groups III and I is not even probable ($P = 0.2$), while the difference between groups III and II is statistically significant ($P < 0.001$). The difference between groups I and II is significant ($P = 0.01-0.001$).

Thus m. l. d. digitotal was less for the animals that did not run on the platform than for those which had to run and for those that served as controls.

Discussion.

Experiments carried out under our test conditions show that muscular exercise increases the tolerance of the guinea-pig for digitalis. It does not seem possible to offer any reliable explanation for this fact at present. Nevertheless it seems probable that the increased flow of blood through the tissues brought on by muscular exercise binds digitalis to the blood and cells far more than rest does. The fact that metabolism is greater when work is in progress may also partly explain the increased elimination of digitalis in the case of muscular exercise.

The experiments also showed that the cumulative action of digitotal-solution in guinea-pigs were slightly pronounced. This was specially the case with the animals that did the running, though it was also apparent in the second group as well. Thus, a considerable number of the animals survived (table 1) after a daily supply for one month of large doses of digitalis, every day

during the last three weeks the lethal dose divided into 2 doses daily.

We have not been able to find any literary statements concerning the cumulative effect of digitalis on guinea-pigs, nevertheless its effect on cat, rat and mouse is considerably greater than our tests on guinea-pigs (LENDLE 1935).

As a cause for the small cumulative effect of digitalis in our experiments could be proposed that the capability of guinea-pigs to absorb digitalis subcutaneously is relatively slight. The animals, however, were rapidly affected by the injections and, to judge by appearance and behaviour, after the same time and in the same degree as after injections of an alcoholic extract of folium digitalis or a pure digitoxinpreparation in the same quantity corresponding to the lethal dose.

On the other hand it seems to be reasonable to suppose that the digitotal-solution used only causes a slight cumulative effect on guinea-pigs in proportion to other digitalis preparations. To support this we refer to our experiments with subcutaneous injections on guinea-pigs of alcoholic extract of folium digitalis and of digitotal tablets and with pure digitoxin under the same experimental conditions, where the cumulative effect was stronger. These investigations supported that the small cumulative effect was not due to a specially low digitoxin content in the digitotal-solution. Control experiments showed that the glycerine in the digitotal-solution did not influence the results (ELMQVIST and RYDIN, 1947, 1).

The rapid elimination of digitalis can also be a cause to the small cumulative effect of the digitotal-doses used (ELMQVIST and RYDIN, 1947, 2).

Our investigations seem to indicate that in certain circumstances the organism can accustom itself to digitalis, and after repeated doses it can tolerate this stimulant better than it did at first. Thus, when the guinea-pigs had received digitalis injections for 9 days, they were less affected immediately after the injections during the ensuing weeks than they were after the first few doses, even though they received twice as much latterly. This observation concerning habituation to digitalis only refers to the visible effect of digitalis during the first few hours after the injection, when the animals were apathetic and weak and showed no desire to move even when touched: it does not refer to the cumulative digitalis effect, which by means of Knaffl-Lenz' tests could be

proved in the animals that had not been exercised. Hence, there is no reason why habituation in this meaning and cumulation should be opposed to each other.

According to certain literary statements the administration of digitalis for any length of time produces a decreased susceptibility to glycosides. For example, UTSONOMIYA (1927) considers that he has established this fact with regard to mice. KLEIN (1915) found a reduction in the salivation reflex in cat after repeated strophanthin injections. He did not interpret this as a typical habituation but as an "Abstumpfung der Organreaktion", as there was no simultaneous reduction in the glycoside storage. It seems to us as if the habituation we have observed in our experiments must be assigned to a similar reaction to that described by KLEIN.

Our experimental investigations on animals should be continued on clinical material. The result seems to indicate that heart patients in movement require more digitalis than they do in bed.

Summary.

Experiments have been carried out on guinea-pigs, which were given daily doses of digitalis subcutaneously for one month. Its effects were followed inter alia by observing the mortality among and the weight of the animals. The minimum lethal dose for intravenous digitalis administration was determined on the surviving animals according to Knaffl-Lenz. The following results were obtained:

- 1) The animals which were immediately exposed to extra muscular exercise after the digitalis injections showed a greater digitalis tolerance than those which did not have to run (tables 1 and 2).

- 2) Under our test conditions it was possible to establish a sort of habituation of the guinea-pigs to digitalis. The strong general effect shown by most of the animals during the first few hours immediately following on the digitalis injections in the first few days gradually subsided.

- 3) During weeks the guinea-pigs could tolerate comparatively large doses of digitalis (half LD 50 doses twice daily). We attribute this to the low cumulative effect of digitotal, the digitalis preparation we used, and to the rapid elimination of digitalis in the guinea-pig.

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From the State Pharmaceutical Laboratory, Stockholm.

Studies on the Destruction and Cumulation of Folium Digitalis Purpureae and Folium Digitalis Lanatae in Guinea-pigs.

By

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The destruction and cumulative action of folium digitalis purpureae and different glycosides or glycoside mixtures of purpurea and lanata drugs has long been the subject of extensive research. In many cases, however, the results are divergent, varying according to the methods adopted and the kind of animals used in the experiments; this is shown by the review articles of LENDLE (1934), WEESE (1938), MOVITT (1946) and others. Among investigations on this subject, we may mention those of OKUSHIMA (1922) and VAN ESVELD (1931, 1932) and especially the studies made by ROTHLIN (1933, 1938), with Hatcher-Brody's technique, showing the comparative cumulative effects of digitoxin, digilanid and strophanthin. According to most experimental investigations on animals, the lanata glycosides have a markedly cumulative action, nearly equal to that of digitoxin, whereas the lanata preparations, on the basis of clinical observations, are often stated to have a weaker cumulative action with less marked secondary effects than the purpurea preparations, but with good therapeutic effect. It seems that, in drawing such conclusions, sufficient consideration had not always been paid to the distinction between the relatively weak cumulative action, but therapeutically favourable effect, on man, and the cumulative, usually highly toxic, effect in the experiments on animals.

The dried leaves of digitalis lanata or galenic preparations thereof have not attracted the same interest as its glycosides or

folium digitalis purpureae. Merely a few investigations on this subject have been published. VAN ESVELD (1931) found in cats a weaker cumulative effect of *lanata* tincture than that of *purpurea* tincture.

Folium digitalis lanatae, however, is now included in *Pharmacopoea Svecica*. The reason why the leaves of *digitalis lanata*, for therapeutic purposes, have attracted less interest than the leaves of *digitalis purpurea* is largely due to the fact that glycosides can be more easily obtained, and in larger amount, from the former. Nowadays, in fact, we endeavour, for therapeutical purposes, to obtain effective and durable glycosides and glycoside mixtures with a constant composition.

From a scientific point of view, however, the authors have considered it to be of interest to institute a comparison between the said two *digitalis* drugs. In our previous determinations of the minimum lethal dose in guinea-pigs according to the Knaffl-Lenz' technique, the *lanata* drug was found to be twice as toxic as the *purpurea* drug. In administration per os to patients with heart disease, however, the same doses of the two drugs were required in order to obtain a fully satisfactory result. In short, no difference whatever in cumulative action or in other respects could be observed between the effect of the two drugs on man (BIÖRCK, ELMQVIST, NYLIN, RYDIN 1946; ELMQVIST, KARNELL, NYLIN, RYDIN 1948).

It might seem natural to try and explain the above-mentioned difference between the results in the animal experiments and in the clinical investigations by the different resorption conditions in the alimentary canal. Such a supposition, however, is gain-said, for example, by ROTHLIN's (1938) observations on the good resorption of *lanata* preparations (*Digilanid*) from the gut, as compared with the *purpurea* glycosides. KWIT, GOLD and CATTELL (1940), on the other hand, found that *Lanatosid C* was not rapidly resorbed after administration per os either to cats or man.

It seemed to us conceivable that the real reason of this discrepancy — at any rate in part — was to be found in different destruction and cumulation conditions. In connection with our previous observations, we accordingly made serial experiments on animals with a view to ascertaining the destruction and cumulation effects of the same *purpurea* and *lanata* drugs as are used in our clinical experiments. As experimental animals, we selected guinea-pigs. Those animals have proved to be particularly suit-

able for digitalis studies, but, so far as we aware, had not previously been used for investigations of this nature.

Experimental.

The experiments were conducted in two series. In the one series the guinea-pigs, in accordance with the procedure suggested by FRAENKEL (1904), daily received a sublethal dose of digitalis, with observation of the mortality. After a certain number of days the surviving animals received an intravenous injection of the same digitalis preparation, in order to determine the lethal dose.

In the other series, in accordance with the procedure proposed by HATCHER and BRODY (1910, 1912), the minimum lethal dose (m. l. d.) of digitalis was determined at certain hours after the animals had received a sublethal single dose of that drug.

The experiments were made on guinea-pigs weighing 400—500 grams. The minimum lethal dose was determined in accordance with Knaffl-Lenz' method, somewhat modified (KÄLLROT and RYDIN 1944).

The alcoholic extracts of folium digitalis purpureae and folium digitalis lanatae used for subcutaneous injections were prepared in accordance with the instructions for alcohol extraction in the Swedish Pharmacopoea Ed. XI, and contained 10 % and 5 %, respectively, of digitalis drug.

In regard to biological effective strength, 1 gram of the purpurea drug used corresponded to 1 gram folium digitalis, international standard, and 1 gram of lanata drug was equivalent to 0.73 gram, Swedish national standard.

The results have been treated statistically according to FISHER (1936) with X^2 and t-analysis.

P denotes probability that different groups belong to the same population.

$P \leq 0.05$ denotes that there is a probable difference between the groups.

$P \leq 0.01$ denotes that the difference is very probable.

$P \leq 0.001$ denotes that the difference is significant.

a) Daily administration of sublethal digitalis dose.

Twenty guinea-pigs received, in a single daily dose, a subcutaneous injection of an alcoholic extract of folium digitalis purpureae in an amount corresponding to 12 mg of the drug per 100 grams of the bodyweight. To twenty other guinea-pigs lanata extract was administered in an amount corresponding to 6 mg of the drug per 100 grams of the body-weight. These dosages amounted to 67 % of the lethal doses. In regard to the surviving guinea-pigs, the lethal dose was determined in accordance with the Knaffl-Lenz' method, on the morning of the day after the last subcu-

taneous injection. The experiments were then repeated, with the following modifications: 20 guinea-pigs received twice daily 50 % of the lethal dose of purpurea extract, 19 guinea-pigs twice daily 50 % of the lethal dose of the lanata extract. The corresponding lethal dose for guinea-pigs who had not previously received digitalis was also determined at that time.

The results are presented in *table 1*.

Table 1.

Results on guinea-pigs receiving repeated doses of alcoholic extract of digitalis subcutaneously.

Groups	Daily dose of digitalis in per cent of m. l. d.	Preparation	Number of surviving animals							Lethal dose on surviving animals mg/100 g.
			Days after injection							
			1	2	3	4	5	6	7	
A	67 × 1	p	20	18	15	8	4			19.4 ± 1.13
B	67 × 1	l	20	20	20	18	18	18	17	9.4 ± 0.31
C	50 × 2	p	20	10	5	3	0			
D	50 × 2	l	19	17	17	17	13	9	7	8.3 ± 0.24
E	0	p								17.5 ± 0.67
										(10 animals)
F	0	l								8.8 ± 0.31
										(10 animals)

p = digitalis purpurea. l = digitalis lanata

The difference in the survival time between the animals that had received digitalis purpurea and lanata, respectively, is significant ($P = 0.001$). On the other hand, no significant difference in the lethal dose between the pretreated and non-pretreated animals could be ascertained. The experiments were conducted during the months of July and August 1945.

b) Administration of a sublethal single dose of digitalis with following m. l. d. determinations.

Two groups of 24 guinea-pigs each received subcutaneous injections of an alcoholic extract of folium digitalis purpureae and lanatae, respectively, in a single dose corresponding to 80 % of the lethal dose after subcutaneous injection. The minimum lethal dose of digitalis purpurea and lanata, respectively, was then determined, in accordance with the Knaffl-Lenz' method, after the lapse of 1, 2, 4 and 6 hours. The experiments were conducted in the month of November 1945.

The following results were obtained:

Fol. dig. purp.

M. L. D.:

1 hour after subc. inj. of 80 % of l. d.	10.6 ± 0.66 mg/100g	(8 animals)
2 hours " " " " " " " "	10.9 ± 0.3	" (4 animals)
4 " " " " " " " "	13.3 ± 0.52	" (8 animals)
6 " " " " " " " "	12.9 ± 0.55	" (3 animals)
for non-pretreated animals	14.7 ± 0.59	" (8 animals)

Fol. dig. lan.

M. L. D.:

1 hour after subc. inj. of 80 % of l. d.	5.5 ± 0.34 mg/100 g.	(8 animals)
2 hours " " " " " " " "	6.9 ± 0.58	" (4 animals)
4 " " " " " " " "	6.2 ± 0.29	" (8 animals)
6 " " " " " " " "	6.2 ± 0.69	" (4 animals)
for non-pretreated animals	7.0 ± 0.36	" (8 animals)

The difference between the lethal dose of digitalis *purpurea* for non-pretreated animals and for the animals pretreated with digitalis is significant for the values after 1 and 2 hours ($P = 0.001$), whereas for the values after 4 and 6 hours it is not even probable ($P = 0.1$).

For folium digitalis *lanata* the difference between the lethal dose for non-pretreated animals and for animals pretreated with that drug is very probable — significant for the 1 hour value ($P = 0.01-0.001$), whereas after 2, 4 and 6 hours the difference between the lethal dose for non-pretreated animals and the lethal dose for pretreated animals is not significant.

Discussion.

With the methods adopted by us, it has been ascertained that alcoholic extract of folium digitalis *lanatae* in guinea-pigs shows a greater destruction and a less marked cumulative effect than folium digitalis *purpureae* (see the tables). These results correspond with the comparison made by VAN ESVELD (1931), in experiments on cats, between tinctures of these two drugs in accordance with the Hatcher-Brody procedure as well as with the clinical reports showing a lower cumulative effect and a more rapid destruction of the *lanata* preparations (MOWITT, 1946). So far as it is warrantable to draw conclusions from animal experiments as to the effect of digitalis on man, our observations on the greater destruction of the *lanata* preparations seem, at any rate in part, to account for the apparent difference in the effects

of the two digitalis drugs in our earlier experiments on animals, on the one hand, and clinical experiences on the other.

In comparison with other kinds of animals, the guinea-pigs shows a particularly rapid destruction of digitalis. Thus, whereas with Hatcher-Brody's procedure in experiments on cats the effect of an administration of digitalis amounting to 50 % of the lethal dose can be seen for at least 5 days as regards the purpurea preparation and for 24—48 hours as regards lanata tincture, a corresponding effect in experiments on guinea-pigs after administration of no less than 80 % of the lethal dose can be found a few hours only. According to HATCHER (1921), the effect of the first-mentioned digitalis dose can be observed for 5—12 days, 2 days, and less the one day as regards cats, dogs and rabbits, respectively, SOLLMANN (1944) contends that this persisting effect of digitalis is the shorter, the more resistant a species is to that drug. Our experiments on guinea-pigs show that this conclusion is not tenable.

Our cumulation tests according to the method of FRAENKEL showed a good cumulative effect for the purpurea drug, as well as for the lanata drug in a somewhat higher dosage. The surviving animals, however, did not show any statistically significant reduced tolerance for digitalis in the Knaffl-Lenz' tests, despite of the high digitalis dose (67 and 50 % of the lethal dose once resp. twice daily) that the animals had received for several days. These experiments likewise show the rapid destruction of digitalis in guinea-pigs. It seems that, under our experimental conditions in testing these alcoholic extracts, no cumulative storage of digitalis in the organism takes place to any appreciable extent. In correspondence with the findings of BAUER and FROMHERZ (1933), the cumulation in this case seems, instead, to be due to pathological changes in the functional or morphological state of the organism, which finally result in the death of the animal. On the other hand, an accumulation of digitalis can be found also in guinea-pigs if the drug is administered for a considerable length of time. This is shown by our experiments with Digitotal (Astra), in which the animals received digitalis twice daily for a month (ELMQVIST and RYDIN, 1948).

The experiments should be supplemented with electrocardiograms, histological examinations of the heart and certain other organs, on the same lines as the experiments conducted by BAUER and REINDELL (1938) on cats and by ROTHLIN on cats and dogs.

It would also be of value to supplement these studies by testing the effects of glycoside preparations (digitalis, digilanid, cedilanid) under the same experimental conditions as in the present investigation.

Summary.

Experiments were made with guinea-pigs on the destruction and cumulation of alcoholic extracts of folium digitalis purpureae and lanatae on the basis of FRAENKEL's procedure as well as that of HATCHER-BRODY.

It was found that the lanata extract was eliminated more rapidly and was less cumulative than the pupurea extract.

The guinea-pigs showed a particularly marked capacity for the destruction of digitalis. The cumulation, under our experimental conditions, does not seem to have been due to any storage of digitalis in the organism.

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On Acclimatization in Connection with Acute Carbon Monoxide Poisonings.

By

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The anamnesis of acute and chronic carbon monoxide poisoning cases often reveal, that the patients during the first days and weeks at work easily get a headache and feel sick or dizzy. But the symptoms soon disappear after a time at work in CO-environment. A kind of acclimatization has taken place, a common phenomenon in toxicology. A driver, for instance, who has become accustomed to CO, can stay in a garage, where a person, who is not acclimatized to it, immediately feels sick. An example of this follows.

One morning our laboratory technician took blood tests of drivers at a garage. While the "acclimatized" drivers felt nothing in the room (the COHb-content of their blood was 18—22 %) the laboratory assistant after a while felt dizzy, got a headache and lost consciousness owing to acute CO-poisoning. The COHb content of her blood was only 19.5 %, examined immediately after the poisoning.

Patients also often complain, that the symptoms of poisoning appear more easily on their returning to work after a weekend or a vacation. The same phenomenon is very common in other poisonings such as nitroglycerin, nitroglycol, trinitrotoluene (TNT) etc. poisonings.

On the other hand we have observed that these poisons also can cause allergic symptoms.

Earlier Observations with Respect to Acclimatization.

In the literature available in Finland we have found very little about studies on acclimatization when carbon monoxide is involved. CAMBELL (1929—1930) reports, that rats, rabbits, mice and guinea pigs after acclimatization tolerate higher carbon monoxide concentrations than before tests. KILLICK (1937) examined persons, who were, in a room where the CO-content was 0.023—0.046 %. She noticed, that they became acclimatized to this low carbon monoxide content. She observed no changes in the blood picture. But at the end of the experiment she found less COHb in the blood than in the beginning. Further, KILLICK observed polycythemia, reticulocytosis and increased blood volume in the acclimatized mice. She points out that some other factor too is involved in acclimatization. NASMIT and GRAHAM (1906) put guinea pigs in CO-atmosphere, in which the COHb content of the blood reached 25 %, whereas the red cells and hemoglobin gradually increased during 4 weeks from 5.88 mill. and 88 (Hb) to 7.96 mill. and 105 (Hb). As the CO content of the blood mounted to 45 %, the red cells also rose to 10.5 mill. and hemoglobin to 110. FOLKESON (1944) demonstrated acclimatization in workers employed in steel mills in conditions where the air contained carbon monoxide. He, too, points out, that the acclimatization depends not only upon polycythemia but also on some "absorption mechanism" in the body. According to German authors, in about 30 % of the chronic CO-poisoning cases polycythemia can be found (SYMANSKI 1936). NORO, however, has not observed distinct polycythemia in automobile drivers, who have had chronic carbon monoxide poisoning.

BRIEGER (1943) examined dogs which during 11 weeks were exposed for 6 hours 6 times weekly to 0.0096 % CO. After 3 weeks he found increase of Hb in five dogs. Hemoglobin reached the maximum after 6 weeks, but after 9 weeks it, as well as the red cells, had diminished to the earlier level.

Thus it has been proved, that the organism can get acclimatized both to great and small CO-content. The phenomenon has been proved to be caused by polycythemia together with some unknown factor.

Questions.

In order to explain the phenomenon of acclimatization accurately, we have tried to get an answer to the following questions:

1. How do rats and mice react to high CO-content, if exposed every day until unconsciousness occurs?

How does the acclimatization develop and in what concentrations?

2. How are acclimatization and polycythemia related?
3. Does any other factor play a rôle in acclimatization?
4. What is the COHb content of the blood during repeated acute poisonings while acclimatization is developing?
5. Do some general toxic symptoms appear after repeated acute CO-poisonings?

The Apparatus and Experiment Technic.

In Figure 1 the apparatus is described.

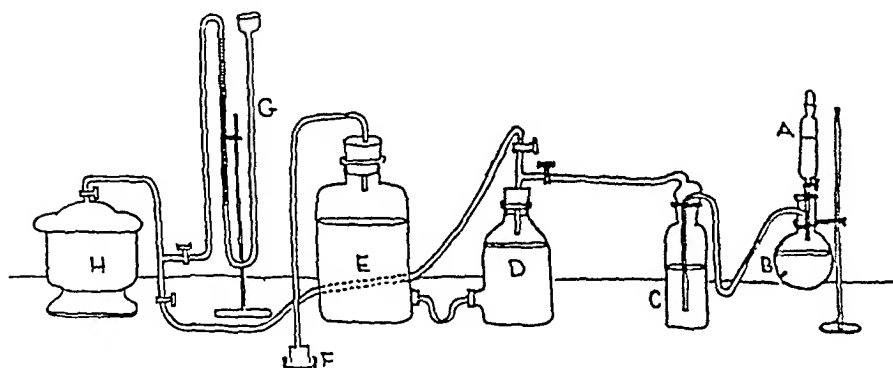


Fig. 1. The apparatus.

Carbon monoxide for the experiments was obtained by dripping formic acid from container A into container B, in which there was conc. sulphuric acid. The latter was heated a little. Carbon monoxide was conducted through concentrated KOH-solution (C), in which the sulphuric acid was absorbed. From there the pure carbon monoxide was conducted into the supply container D, which is in connection with a bigger bottle (E), containing water. With an air pump F the air was pumped into E, whereat the pressure both in E and D increased. The gas was conducted from D into gasbyrette G. From this the required amount of gas was conducted into exsiccator H, where the test animal was. When there was little under-pressure the conducting of CO into it was easy.

The blood tests were taken as follows: A wound was cut in the blood vessel of the tail with a knife. The rat was put into bottle without a bottom, in place of which there was a removable tin lid with a hole for the tail of the rat. Fig. 2.

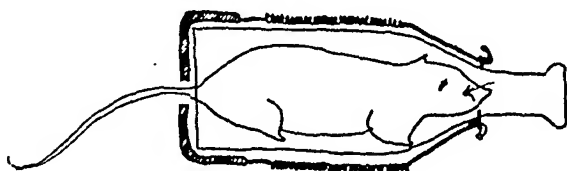


Fig. 2.

Technic of the Experiments.

The experiments were performed as follows: The test animal in the exsiccator was in the given CO-content (1 %, 0.5 %, 0.4 % or 0.25 %) until it lost its consciousness. However we did not wait until full unconsciousness was reached but for the moment, when the animal could not move any more when put on a sloping surface in the vessel. The interval between the beginning of exposure and the moment of unconsciousness was marked.

We made our experiments with 20 rats and 47 mice. The average weight of the rats was 260 gr and the mice 28 gr.

1. The Acclimatization of the Test Animals in Different CO-Contents.

In Figure 3 the graphic curves of acclimatization of three rats in CO-contents of 1 %, 0.5 % and 0.4 % are given.

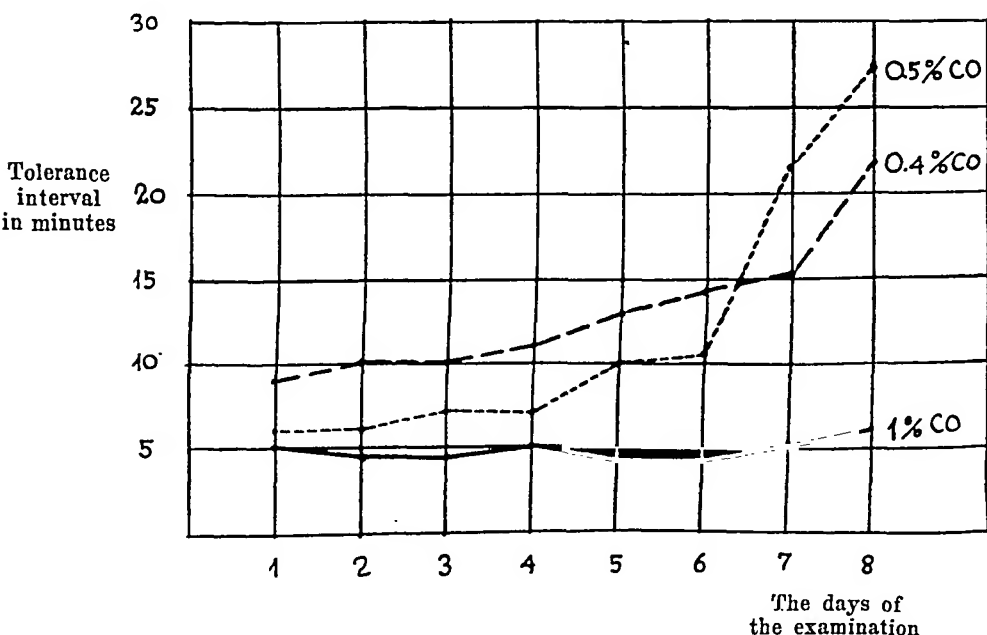


Fig. 3. The increase of the carbon monoxide tolerance in different CO-contents.

(On the axis of ordinates: The interval between tolerance (from beginning of the experiment to unconsciousness). On the axis of abscissas: The days of the examinations.)

The curves indicate:

If the CO-content was high (1 %), the test animals lost consciousness after about 5 minutes. In eight days only insignificant acclimatization could be observed.

If the CO-content was 0.5 %, already on the third day distinct increase of the interval of tolerance was observed, and on the eighth day it was about four times that of the first experiment day.

In CO-content of 0.4 % the same result could be seen. However, it can be noted in Fig. 3 that individual variations among test animals were observed. (One animal could not tolerate 0.4 % content as well as the other 0.5 % content.)

The same experiment was made with 40 mice and 11 rats. The results in general corresponded with those given in Figure 3.

Summary: The test animals (rats and mice) can get very well acclimatized to carbon monoxide in 0.5 % and smaller contents. It is uncertain whether acclimatization to 1 % content can develop.

2. The Effect of an Intermission in Exposure on Acclimatization.

In order to get an answer to the question whether an intermission in exposure has any effect upon acclimatization, we made the following experiments:

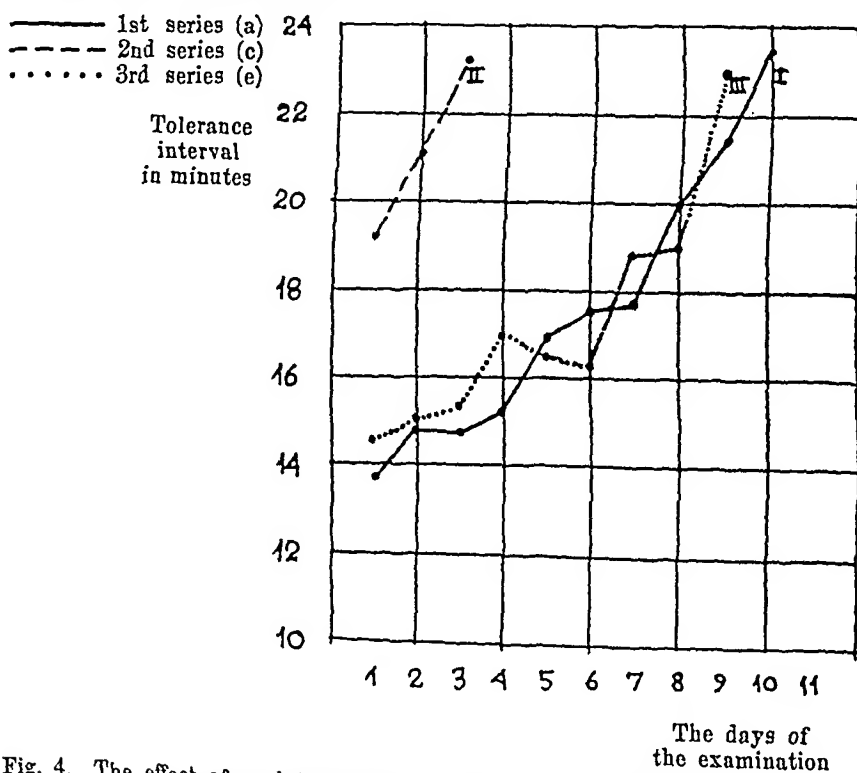


Fig. 4. The effect of an intermission in exposures on acclimatization to carbon monoxide contents of 0.5 % (rat).

- a. The test animal was exposed daily during 10 days to carbon monoxide (0.5 %). (The first series in Figure 4.)
- b. An intermission of 4 days followed.
- c. After this intermission the animal was again exposed for 3 days. (The second series in Figure 4.)
- d. After this exposure an intermission of 8 days followed.
- e. After the second intermission the test animal was exposed again for 8 days.

The results of these experiments are to be seen in Figures 4 and 5.

In the first series of experiments the tolerance of test animal rose (rat) during 10 days from 13 min. 45 sec. to 23 min. 30 sec. After an intermission of 4 days the tolerance interval diminished from 23 min. 30 sec. to 19 min. 10 sec. *The test animal had consequently lost some of its tolerance acquired during the 4 days.*

When the exposure was continued, the animal reacquired its earlier tolerance interval (23 min. 15 sec.) in 3 days. After this an intermission of 11 days followed and the increased tolerance again diminished to 14 min. 35 sec., when the third series was started. During the longer intermission the animal had lost more of its tolerance than during the *shorter one*. By the ninth day of the 3rd series the animal had got back its earlier tolerance.

A similar result was obtained with mice (Figure 5).

In the first series of the experiment the tolerance interval of the test animal rose during 9 days from 15 min. to 48 min. After this an intermission of 8 days was kept. On the first day following the intermission (2nd series) the tolerance interval was 24 min. (Thus it had diminished from 48 min. to 24 min.) After 7 days' exposure the tolerance interval rose to 70 min. Then an intermission of 4 days was kept, on the first day subsequent to which (3rd series) the tolerance time was 44 min. At the end of the third series tolerance interval had increased to 90 min.

The same experiment was made with 7 rats and 7 mice. The results obtained corresponded in general to those described above. However, it could be observed, that the diminishing of tolerance during equally long intermissions varied individually. Thus it was observed for instance that in one case the tolerance diminished (0.5 % CO) during an intermission of 10 days from 17 min. to 10 min. 30 sec., whereas the tolerance interval in another case in the same conditions diminished from 13 min. to 12 min.

c. After the intermission the exposure was continued on 11 successive days. On the first and the last day the red corpuscles and hemoglobin were examined again.

The results are presented in Table 1.

Table 1.

The red blood count of a rat during acclimatization in CO-contents of 0.5 %.

Day of experiment	Interval of tolerance	Red corpuscles	Hemoglobin
1.....	10 min.	7.5 milj.	70
2.....	9 »		
3.....	9 »		
4.....	9 $\frac{1}{2}$ »		
5.....	10 $\frac{1}{2}$ »		
6.....	12 »		
7.....	12 »		
8.....	12 »		
9.....	14 »		
10.....	17 »	9.0 milj.	100

Intermission of 10 days.

1.....	10 $\frac{1}{2}$ min.	8.4 milj.	92
2.....	13 »		
3.....	13 »		
4.....	12 $\frac{3}{4}$ »		
5.....	12 $\frac{10}{12}$ »		
6.....	11 »		
7.....	11 $\frac{1}{4}$ »		
8.....	13 $\frac{3}{4}$ »		
9.....	12 »		
10.....	14 $\frac{5}{12}$ »		
11.....	15 »		

It can be noted that:

a. In 10 days a rat acquires distinct polycythemia at the same time as tolerance of carbon monoxide grows.

b. The polycythemia diminishes simultaneously with the diminishing of tolerance.

The same experiment was performed on 8 rats, and the results corresponded with the foregoing.

The experiments show, that in acclimatization there is a clear correlation between the rise of the tolerance and polycythemia.

But there is also another factor in acclimatization which is revealed in Table 1. In the beginning of the experiments, the tolerance interval was 10 min. Er. 7.5 mill. and Hb 70. After exposure

of 10 days the interval of tolerance was 17 min. Er. 9.0 mill. and Hb 100. But after the intermission Er. and Hb were still 8.4 mill. and 92, although the tolerance interval was only 10 min. 30 sec. If increased tolerance were dependent only on polycythemia, one could expect a longer interval of tolerance for in the beginning of the experiment, when Er. was 7.5 mill. and Hb 70, the time of tolerance already was 10 min.

Similar observations have been made in 5 cases.

This shows that in addition to polycythemia other factors also play a rôle in acclimatization, or that polycythemia is only a secondary phenomenon in connection with it.

As we mentioned in the literature survey, BRIEGER demonstrated in experiments with dogs, that polycythemia, which appears during carbon monoxide exposure, disappeared if the time of the exposure was long enough.

We made an experiment, similar in principle, with three rats and observed the same results: After 77 days' exposure the red corpuscles and hemoglobin counts after having increased, returned to their previous values. In this experiment, however, after 77 days exposure, there was no polycythemia, but the tolerance had clearly increased.

This experiment too proves, that polycythemia is not the only factor in acclimatization. It may as well be a secondary phenomenon.

4. COHb during the Acclimatization.

In this series of experiments the rats were put for 11 min. into the CO-contents of 0.5 % on 47 successive days. The results are given in Table 2.

Table 2.

COHb, the interval of tolerance, Er. and Hb in rats. (COHb-test was taken the day after tolerance interval was determined.)

Day	Duration of exposure and tolerance interval ¹	Er.	Hb	COHb
1	11 min.	8.6 millj.	90	28.7
¹⁰	13 »			
¹¹	11 »	9.3 »	102	28.5
²⁴	18 »			
²⁵	11 »	8.5 »	108	34.0
⁴⁶	23 »			
⁴⁷	11 »	8.1 »	98	27.1

¹ The tolerance interval was determined on the 10th, 24th and 46th day.

Similar experiments were made with 8 rats. The results were in general the same.

In Table 2 we can see that, in spite of the fact that the tolerance was rising, the COHb remained about the same.

When comparing Table 2 and the Figures 3, 4, 5 and Table 1, also the following observations can be made.

If the exposure has been "ad maximum", *i. e.* (Figure 3, 4, 5 and Table 1) until unconsciousness occurs, the acclimatization takes place faster than if the exposure is of shorter duration (Table 2).

General Toxic Symptoms during and after Acclimatization.

During the experiments the general condition of the test animals became worse. They were not so lively as the control animals and they were lying most of the time. If the test animals were pregnant, when exposed, the young they bore died within 2 days. Of 7 parturitions with litters of 6—8 young, only two remained alive. An animal which was exposed during pregnancy bore 8 young. All died within 4 days. After this the animal was not exposed to carbon monoxide and after 1½ months it bore a litter of 6 young, which all remained alive.

In similar experiments Dr. RAEKALLIO and RITAMA have also made histological examinations and observed clear changes in the brains of animals.

We can say that this "acclimatization" has not occurred without injuries to the test animals.

Summary.

The authors have exposed rats and mice daily to carbon monoxide in contents of 0.25, 0.4, 0.5 and 1 %. Their observations are as follows:

— The test animals became acclimatized to carbon monoxide in contents of 0.25—0.5 % and the tolerance against CO in 8—15 days increased 2—4 times its former value. In 1 % CO only slight acclimatization was observed.

— An intermission of several days in exposure diminished the tolerance. The longer the intermission was the more tolerance (got by acclimatization) the animal lost.

— In connection with acclimatization polycythemia was observed which disappeared later. However, tolerance clearly increased without polycythemia.

— No decrease of COHb was observed in connection with acclimatization.

— The general condition of test animals became worse during "acclimatization".

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From the Nobel Institute for Neurophysiology, Karolinska Institutet,
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The Form Variations of the Spike Recorded by a Micro-electrode Applied onto the Mammalian Retina.

By

BO GERNANDT.

Received 23 October 1947.

When recording from the cat's retina by the standard micro-electrode technique of this laboratory (see *e. g.* Summary by GRANIT, 1947) one often observes that the isolated elements fall into two categories, (i) the typical large spike and (ii) another smaller spike which also appears to be thinner. For several reasons it was decided to expand the record of the spikes by using the horizontal sweep circuit of the cathode ray tube and running the film vertically past it so as to obtain a number of fast pictures of various types of elements. Most fibres isolated by the micro-electrode are spontaneously active so that a continuously moving sweep circuit will every now and then catch a spontaneous discharge travelling past the electrode; if not, a discharge can always be obtained by illumination. Fig. 1 illustrates how these events are reproduced by our technique of recording. The sweep movement appears as a horizontal pattern of lines occasionally interrupted by a spike.

All the experiments were carried out on fully dark adapted decentered cats which, in order to eliminate eye and head movements, had received some 3—5 ml of a 20 % urethane solution. The nature of the element isolated, whether a pure on-, and on/off- or a pure off-element, was determined by illuminating the eye with light of wave-length 0.510μ from our Wright colorimeter (WRIGHT 1946). The microelectrode was inserted in the usual fashion after removal of cornea and lens.

Results.

Photographs were taken of 44 on/off-elements, 13 pure on-elements and 7 pure off-elements.

Since the aim of the investigation was to find out whether the two types of spike observed could be correlated with any of the

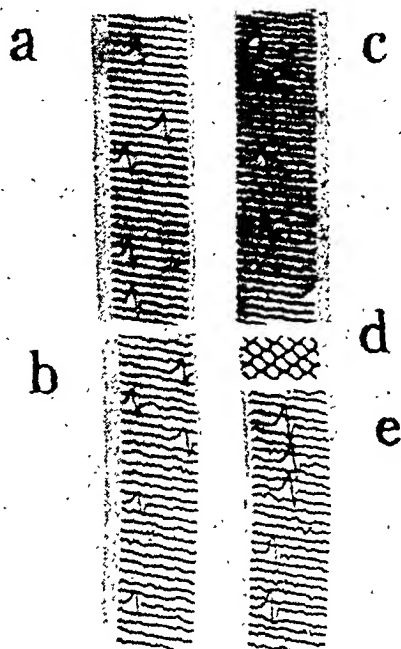


Fig. 1. Micro-electrode. Cat's retina. Fast sweep record of four separate spikes, *a*, *b*, *c* and *e*. Sweep speed shown in *d* by oscillation of 500 Hz.

known *physiological* attributes of such spikes no attention was paid to correcting for the distortion caused by the polarisability of the platinum micro-electrode (which has condenser properties) as well as for the use of the exceedingly small coupling condensers which are necessary in order to remove the large deflexions caused by the electroretinogram. However, the electrical recording system was calibrated through the animal with the micro-electrode on the retina. Thus the calibrations in fig. 2 include the sum total of distortions to which a square wave current will be subjected in our recording system. The latter consists of micro-electrode (a fine platinum tip insulated by glass) eye of animal, condenser coupled amplified and cathode ray tube.

Fig. 1 illustrates four separate experiments and between the last two a calibration of sweep speed with a 500 Hz wave (the tops of which touch one another). The four impulses illustrated are much alike. The spikes vary in size from experiment to experiment between 0.100 to — occasionally — 0.300 mV. Smaller spikes than that cannot be used for analytical work because adjacent impulses interfere too much with the measurements. In order to

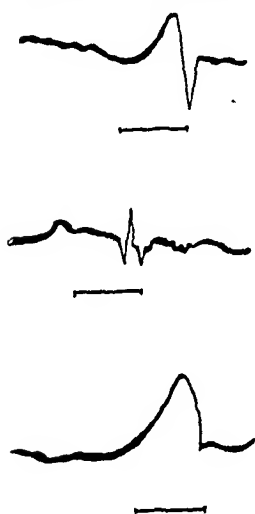


Fig. 2. Magnified records obtained as in fig. 1. *a*, typical spike; *b*, atypical spike; *c*, calibration to 0.200 mV. Time: 2 milliseconds.

- a* analyze the properties of a spike it is necessary to be able to diminish amplification until only one spike stands out alone as in the records of fig. 1. This cannot be done with spikes below about 0.100 mV.

In fig. 2 a calibration, a normal, and an atypical spike have been magnified and reproduced together for comparison. The spike begins with a fast, positive phase, then follows a slower negative phase and a very small third positive phase. The atypical element appears as a brief negative phase between two small and still briefer positive deflexions. The negative phase is of the order of 0.050—0.075 mV. Whereas in the normal spike the negative phase lasts about a millisecond, its duration is only about half a

millisecond in the atypical spike. All the atypical spikes belonged to on/off elements.

It is my impression that the spikes increase in size when the micro-electrode is shifted away from the blind spot and diminish when it is shifted towards it.

The normal or typical spike has a negative phase which seems too expanded to be due wholly to nerve activity. Probably it contains an electrotonic component from the cell bodies, suggesting that the selection of a large spike for analysis consists in shifting the microelectrode in the direction towards the ganglion cell. The fast spike, atypical from the point of view of analytical usefulness, has the characteristic triphasic form of the action potential of a fibre lying in a conducting medium.

Comment.

For some time now a great deal of analytical work has been carried out in this laboratory with elements isolated by the micro-electrode technique in the eye of the cat. By various methods the spikes have been shown to have different physiological properties, particularly with respect to tests with illumination and polarization. The detailed inspection of the 64 spikes investigated by the »fast sweep» recording technique leaves no doubt about the fact that the material collected here during the last years, has been extremely homogeneous from the point of view of general appearance of the spike. Not only is the atypical spike rare, but it is too small for analytical work. In the course of a lengthy experiment the spike mostly diminishes; if for no other reason, then because of the slow gradual change of position caused by the respiratory movements. It is therefore necessary, at the outset, to have a large spike. This condition imposes a principle of selection on the material. For this reasons, too, it is satisfactory to be able to affirm with confidence that the selection according to analytical minimum size has had no influence on the appearance of the spike. The spikes used for analytical work must all have been practically alike and can merely have varied in amount of potential. Also, if an on-element consisted of a single, an on/off-element of a double fibre, then, in the latter case, the synchronization of the two fibres must be so perfect as to suggest some kind of histological connexion maintained up to the next station in the external geniculate body.

It would not seem unreasonable to assume that on/off-elements are due to some kind of twin fibres. But this assumption, if true, cannot be maintained on the basis of a chance combination due to insufficient isolation by the micro-electrode. If there be "twin" fibres, the "twin" system must be a physiological property organized by the retina. Otherwise it is very difficult to understand why the on/off-spikes never show any sign of falling apart so as to form double spikes. This, of course, is no decisive argument against the idea of spikes being due to perfect synchronization.

Summary.

Fast recording of the spikes isolated by a micro-electrode applied onto the cat's retina shows that, as soon as the spikes are

large enough for an analytical work, they are all alike though varying in size from 0.100—0.300 mV, The form of the spike is illustrated in fig. 1. It suggests that it consists of an optic nerve impulse combined with an electrotonic component from the cell bodies.

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Differences between Autonomic and Somatic C fibres to Stimulation with Constant Currents.

By

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It is well known that the unmyelinated nerve fibres — C fibres — have partly an afferent (RANSON and BILLINGSLEY, 1916, ZOTTERMAN 1933, 1936, 1939, CLARK, HUGHES and GASSER 1935 and others) and partly an autonomic efferent function (CHASE and RANSON 1914, BISHOP and HEINBECKER 1930 and others). Most of the electro-physiological data concerning C fibres have been obtained from studies of the vegetative C fibres in visceral nerves (BISHOP 1934, GRUNDFEST and GASSER 1938, BISHOP and O'LEARY 1939, and others) since in certain of these nerves observations are not distorted by the presence of other fibre types. For the same reason, however, comparatively little is known of possible differences between afferent and efferent C fibres. C. v. EULER (1947) has recently shown that there is a definite difference in the thermal sensitivity between these two categories of fibres. The afferent C fibres are sensitive to local heating a few degrees above body temperature while the efferent fibres do not discharge in response to this type of stimulation. In this work I have compared the discharge of afferent and efferent unmyelinated fibres to stimulation with long lasting constant currents.

Method.

The experiments have been performed on decerebrated and decapitated cats, the latter being ventilated with the aid of a Starling pump.

Repetitive discharges from a nerve have been studied by recording the reaction of the respective effector organs. Impulses in the vagus nerve have also been recorded with the aid of a 4-stage amplifier and a cathode ray tube.

In order to record the movements of the stomach, a rubber tube was introduced through a cervical oesophagotomy after ligation of the pylorus. The stomach was washed out with Ringer at body temperature. The tube was connected to a piston recorder. The stomach and about half the tube was filled with Ringer (BROWN and McSWINEY, 1932).

Vasodilatation in the hind limb was recorded by means of a pletysmograph. The upper part of the thigh was shaved and the pletysmograph sealed with vaseline. Changes in volume were registered by a piston recorder, the system being pulse sensitive.

The stimulating device was made up of a commutator, a 50,000 Ω resistance in series with the preparation and a galvanometer for measuring the current strength. In a few cases a stimulator generating rectilinear and rectangular currents was used. This instrument has been described by SKOGLUND (1942). The principle of the experiment depended somewhat on whether the nerve response or the response of the effector organ was used. In the former case the discharge to a rheobasic current could be determined and the effect of an increase on its strength directly observed. With the effector organ as index, however, the rheobasic strength could only be determined by repeating the square-wave stimulus of long duration (not less than 10 msec.). The effect of this was then compared with the effect of increasing the strength of a long-lasting constant current.

The stimulating electrodes were of the silver-silverchloride (Ag-AgCl) type. The cathode was always placed nearest the recording electrode or the effector organ.

Heating of the dorsal roots was performed with the thermal technique employed in this laboratory (see C. v. EULER 1947).

Results.

Efferent C fibres in the vagus nerve. According to CHASE and RANSON (1914) and HEINBECKER and O'LEARY (1933) and others, the vagus nerve in the lower part of the thorax consists almost entirely of unmyelinated nerve fibres and of these only very few are afferent.

If the vagus was severed in the neck and the peripheral stump stimulated with a single sufficiently strong square-wave shock (the threshold varied around 0.7 mA), a single C potential (conduction velocity about 1 m/sec.) was obtained from the vagus immediately above the diaphragm.

On the other hand it was impossible, when stimulating with a constant current of long duration, to elicit a repetitive impulse

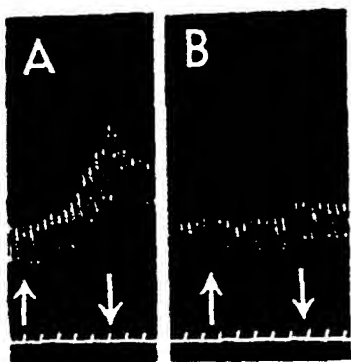


Fig. 1. Decerebrated cat. Recording of gastric tone, during stimulation of the peripheral end of the vagus nerve in the thoracic region. A. Square-wave shocks 0.8 mA, frequency 8/sec. Contraction of the stomach. B. Constant current 2.0 mA. No effect. 5 sec. intervals.

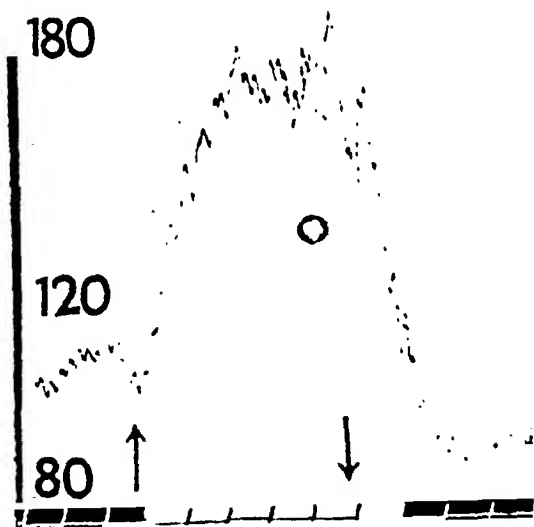


Fig. 2. Decerebrated cat. Recording of blood pressure, during stimulation of the central end of the popliteal nerve. Direct current with increasing strengths up to 0.8 mA. Pressure in mm Hg. 10 sec. intervals.

discharge, observable on the cathode ray tube, even if the current strength was increased to 4 mA or more than 5 times the rheobase.

This lack of sensitivity to polarising currents in the unmyelinated efferent fibres of the vagus nerve could also be observed when recording the motility of the stomach. Stimulation of the vagus nerve in the chest region with sufficiently strong square-wave shocks (frequency about 8/sec.) provoked contraction of the stomach (Fig. 1A). Since the duration of the shocks was long, the liminal current strength (0.5–0.6 ms.), necessary to obtain this effect, can be said to be the same as the rheobase for these fibres. With constant current no change in the motility of the stomach could be recorded, even when the current strength was increased to 2.0 mA, *i. e.* 3 or 4 times the rheobase (Fig. 1 B).

Afferent C fibres originating in the dorsal root. Nociceptive reflexes are transmitted by thin myelinated fibres and by unmyelinated C fibres (see GASSER 1943). Stimulation of the peripheral stump of the severed popliteal nerve with galvanic current regularly produced nociceptive reflexes with marked increase in blood pressure. Even with a current strength of 0.2 mA a slight increase in blood pressure was evoked which increased with the

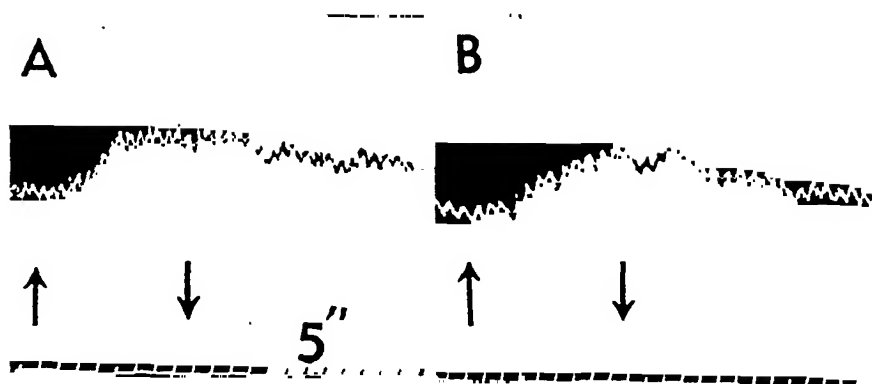


Fig. 3. Decerebrated cat. Pletysmograph on right hind limb, during stimulation of the peripheral stump of the severed dorsal roots L_7 and S_1 . A. Direct current increasing up to 0.7 mA. B. Square-wave shocks of 0.7 mA. 5 sec. intervals.

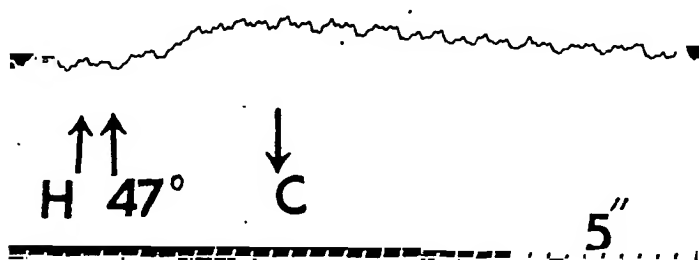


Fig. 4. Decerebrated cat. Pletysmograph on the right hind limb. The peripheral stumps of the severed dorsal roots L_7 and S_1 placed in a thermode. H heated to 47°. C cooled to 37°. 5 sec. intervals.

current strength and reached a maximum between 1.2 and 1.5 mA cathodal stimulation (Fig. 2). This effect is, however, not a pure C fibre effect since also the δ fibres have the same function.

According to HINSEY and GASSER (1930) and BISHOP, HEINBECKER and O'LEARY (1933) only C fibres conduct antidromic vasodilatation, released at the periphery by stimulation of the peripheral end of the cut posterior root (STRICKER, 1876).

Stimulation with a sufficiently strong constant current of the peripheral stump of the L_6 , L_7 and S_1 dorsal roots, placed on the same electrode, elicited a marked vasodilatation (Fig. 3A). The

liminal current strength, was between 0.5 and 0.7 mA. With repetitive stimulation (frequency 4—6/sec.) using square-wave shocks, the threshold was around 0.4—0.6 mA, and thus the same or insignificantly lower than in stimulation with constant current (Fig. 3B).

Experiments have been performed with local heating of these roots using a thermode (BERNHARD and GRANIT 1946, C. v. EULER 1947, and GRANIT and LUNDBERG 1947). Local heating up to 47° elicited definite vasodilatation in the hind limb, which is in accord with GRÜTZNER's (1878) result showing that local heating of the peripheral stump of the severed sciatic nerve produced peripheral vasodilatation.

Comments.

The experiments with the unmyelinated efferent fibres in the lower part of the vagus nerve have shown that these fibres do not respond with a repetitive impulse discharge when stimulated with constant currents (cathodal polarisation) even when the current strength is increased up to 4 times the liminal strength used in repeated stimulation with square-wave shocks of long duration (rheobase). According to C. v. EULER (1947) neither are these fibres sensitive to thermal stimulation despite the fact that local heating produces a local heat potential (relative to a region at normal temperature).

The nociceptive blood pressure reflex which could be recorded from the central stump of the popliteal nerve, stimulated with a constant current, is probably partly transmitted by thin myelinated fibres. The relatively low threshold, 0.2 mA, would seem to indicate this. Since, however, the number of such fibres is relatively small in comparison to the number of C fibres (in the ratio of about 1/10, see C. v. EULER 1947) and since the effect is so marked, there is good reason to assume that the afferent C-fibres also are involved. This has been definitely confirmed in the experiments with stimulation of the dorsal roots. Stimulation with constant current of the peripheral ends of the severed dorsal roots produced a marked vasodilatation. According to HINSEY and GASSER (1930) and others this antidromic stimulus effect is transmitted by the C fibres. The threshold necessary to obtain this effect was the same or insignificantly higher when stimulating with constant current as compared with frequent square-wave

shocks of long duration (rheobase). These experiments show that the afferent unmyelinated C fibres originating in the dorsal root are far more sensitive to constant currents than the efferent C fibres in the lower part of the vagus nerve. Yet the rheobase for the latter fibres appeared to be only insignificantly higher than for the former. These results can also be expressed so that the accommodation of the afferent C fibres »is broken down» (BERNHARD, GRANIT and SKOGLUND 1942) very rapidly when the strength of the polarising current exceeds the value for the rheobase, while that of the efferent C fibres in the lower part of the vagus nerve could not be broken down by polarising currents of more than 4 times the strength of the rheobase (cf. KATZ 1936). It is probable then that the observed differences insensitivity to polarising currents between the afferent and efferent unmyelinated fibres and possibly also their differences with regard to thermal irritability, can be referred to accommodation. A similar difference in accommodation has previously been shown to exist between thick myelinated afferents and efferents, on frogs by ERLANGER and BLAIR (1938), on cat by SKOGLUND (1942) and on man by KUGELBERG (1944).

Summary.

Afferent and efferent unmyelinated fibres have been compared with respect to their tendency to react with repetitive impulse discharges to stimulation with constant currents.

Stimulation with constant currents of the efferent C fibres in the lower part of the vagus nerve of the cat does not initiate a repetitive discharge with strengths up to more than 4 times the rheobase.

On the other hand C fibres of dorsal root origin are extremely sensitive to stimulation with constant currents of just supra-rheobasic strength.

Peripheral vasodilatation can be elicited by local heating of the peripheral stump of the dorsal roots.

The difference between the afferent and efferent C fibres with respect to stimulation by constant currents agrees with their differential thermal sensitivity.

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Changes in the Carbon Dioxide Combining Power after Injection of Dialyzed Casein Digest.

By

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In the production of amino acid preparations intended for intravenous injection, one is faced with the question of a suitable pH. Owing to the different methods adopted for the production of amino acid preparations of the caseinhydrolyzate type, the pH-values in the different preparations vary considerably. As the amino acids have a marked buffer capacity, a change of the pH-value signifies that large amounts of acid or base are bound. This involves the risk that an amino acid solution with an unsuitable pH-value may entail considerable changes in the CO₂ combining power after intravenous injection.

HOPPS and CAMPBELL (1943) showed that if an amino acid solution (enzymatic casein-hydrolyzate) with a pH-value of 4.6 is mixed in vitro with plasma in amounts corresponding to an intravenous infusion of 1,500 ml of a 10 % solution in man, a fall of the CO₂ combining power from 60.9 to 6.9 volume per cent will result. In two cases, however, after infusion of 1,500 ml of the same 10 % amino acid solution for 8 hours, the CO₂ combining power was found to have merely fallen from 59.2 and 58.7 to 50.5 and 52.6 volume per cent., respectively, which they presume to be due solely to dilution of the blood volume. These experiments indicate that even a rather acid solution of amino acids in some way or other is rapidly neutralized by the body.

In an investigation by LEARNER, ROBINSON, GREISHEIMER and OPPENHEIMER (1945) on the effect of an enzymatic casein digest

(amigen) on the intestinal motility, although the solutions had a pH-value of 5.0, no fall of the CO_2 combining power was observed. The amount injected was 6.5 ml of a 10 % solution per kg of the body-weight, and the rate of injection was 6—16 ml per minute.

In order thoroughly to study the changes in the CO_2 combining power after injection of a dialyzed enzymatic casein digest, the investigation reported below was made. In this study, the changes in the CO_2 combining power were placed in relation to the acidity of the solutions. No direct linear relation between the pH-value of the solutions and the changes in the CO_2 combining power of the blood is to be expected: the change is directly proportional to the amount of acid or base required in order to shift the pH-value in the amino acid mixture. As shown in the electrometric titration of the amino acids, the buffer capacity at the different pH-values is subject to great variations. For this reason, the change in the CO_2 combining power cannot be computed directly from the pH-value of the solutions without knowing the titration curve of the substance.

In the enzymatic hydrolysis (trypsin-erepsin) of casein, it is necessary, in order to maintain an optimal pH-value, to add alkali. For this purpose, sodium hydroxide is employed. As the amino acids in the casein-hydrolyzate are synthetized to protein in the organism, one may expect to obtain an excess of alkali, which, in turn, will entail an increase of the CO_2 combining power. In order to determine the excess of alkaline metals in the casein-hydrolyzate here tested, the amino acids were burnt, whereupon the ash was titrated.

Experimental.

Preparation. In these investigations, a dialyzed casein hydrolyzate, Aminosol,¹ which has been described by WRETJÖND (1947), was employed. The concentration of the preparation was 25 %, which corresponds to 32.7 mg nitrogen per ml. The pH-value of the solution was 6.87.

1. Electrometric Titration of Aminosol.

Method. For the determination of the pH-value, a glass electrode² was employed. The accuracy of the determinations was within 0.005 pH. The titration was made on 5 ml of 25 % Aminosol with 1.00 N HCl

¹ Aminosol is made by Vitrum, Stockholm.

² The glass electrode and potentiometer were of the type PHM 3, Radiometer, Copenhagen.

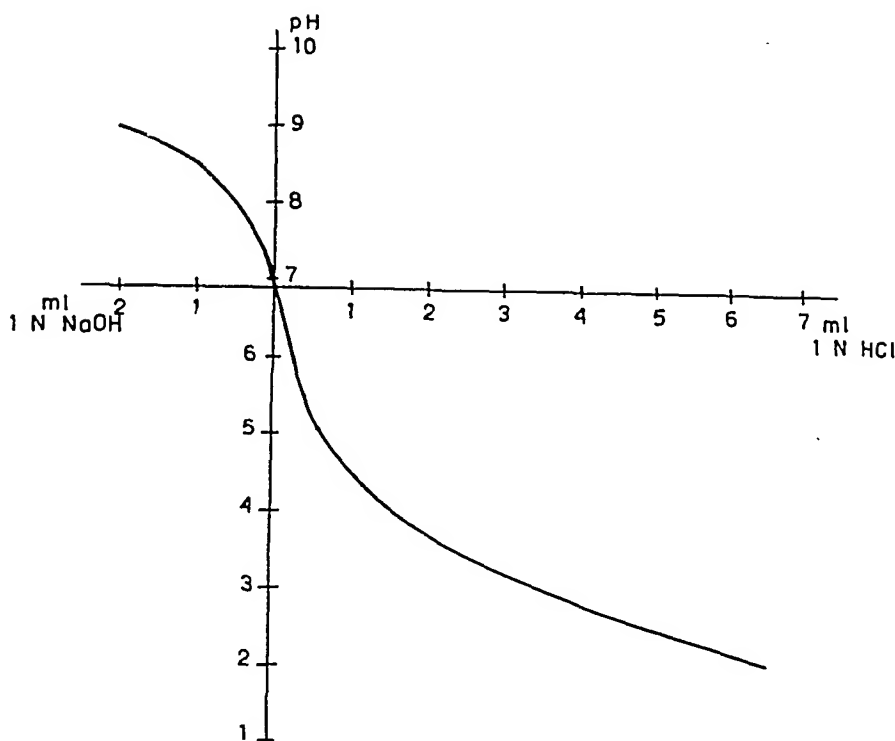


Fig. 1. *Electrometric titration of dialyzed enzymatic casein digest.* The ordinate shows the pH-value. The abscissa shows, to the right of the origo, the amount of 1.00 N HCl added, and to the left the added amount of 1.00 N NaOH. The titration was made on 5 ml of 25 % Aminosol.

and 1.00 N NaOH. After each addition of 0.1 ml acid or base, respectively, the pH-value was determined. In these tests the temperature was 23° C.

The result of the titration is seen from the curve in Fig. 1. The curve clearly shows that the minimum buffer capacity lies at about pH 6.5. An addition of 1 ml N HCl in this case will shift the pH-value by 2.1, whilst an addition of 1 ml 1 N HCl at a pH-value of 4.5 will shift the value by 0.75.

The curve also indicates that 1 g Aminosol (dry) with a pH-value of 6.87 binds 2.64 ml of 1 N HCl before pH 3 is reached.

2. Intravenous Injection of Aminosol with Different pH-Values.

Method. Rabbits (weighing 1.6—3.1 kg) were employed. The amount injected was 5 ml of 2.5 % Aminosol per kg of body-weight. In order to obtain solutions of different pH-value, the original solution was mixed with 1 N HCl and 1 N NaOH, respectively.

Blood samples (1-2 ml) were taken from the rabbit's ear with an ordinary syringe, after dilating the blood-vessels with xylene. The point of the cannula was inserted in the vein in the direction of the ear-tip and, on cautious aspiration, 1-2 ml of blood was easily obtained. The blood was introduced into small centrifugal tubes containing sodium oxalate powder (5 to 10 mg) and was immediately centrifuged, whereupon the CO_2 combining power was determined according to the method of PRYNE and VAN SLYKE (1932) in 0.2 ml of plasma. Double determinations were made and the difference between them was always found to be less than 0.5 %.

Samples for the determination of the CO_2 combining power were taken immediately before the injection of the exten hydrolyzate, which was made during 30-45 seconds. Exactly 5 and 30 minutes after the beginning of the injection new samples were taken. The total number of rabbits subjected to this test was 21. The distribution of the animals according to the pH-value of the solutions and the added amount of acid or base is tabulated below:

2 animals pH 8	0.5 ml 1.00 N NaOH per 5 ml 25 % Amino-	Acid
4 " " 6.87	" " " "	" "
3 " " 5.33	0.5 ml 1.00 N HCl	" "
3 " " 4.85	0.75 " " " "	" "
3 " " 4.16	1.25 " " " "	" "
3 " " 3.76	2.00 " " " "	" "
3 " " 2.75	4.00 " " " "	" "

The change in the CO_2 combining power in the sample after the lapse of 5 and 30 minutes was then computed in percentage. The percentage differences thus obtained were plotted in Fig. 2 (the 5 minutes values) and Fig. 3 (the 30 minutes values) as ordinates in a system of coordinates where the abscissa denotes the amount of 1 N sodium hydroxide and 1 N hydrochloric acid, respectively, with which 5 ml of the 25 % Aminoacid had been mixed.

As above indicated, the effect on the CO_2 combining power should, theoretically, be directly proportional to the amount of acid or base supplied. It seems therefore desirable to make a statistical analysis of the relation between the change in the carbon dioxide combining power and the amount of acid or base added and to calculate the regression line for the connection between them. (BONNIER and TERN 1940.)

Table I.

	Mean value	Regression coefficient	Correlation coefficient	Regression line equation	Mean squares within rabbits
5 min. after injection	$x = 1.16$ $y = -3.34 \pm 2.13$	-6.14 ± 0.77	-0.878 ± 0.050	$y = 3.78 - 6.14x$	37.08 ($\sigma = \pm 6.09$)
30 min. after injection	$x = 1.16$ $y = 2.28 \pm 2.01$	-4.85 ± 1.03	-0.735 ± 0.10	$y = 7.91 - 4.85x$	

The result of the statistical analysis is seen in Table I, where it is clearly shown that there is a significant correlation. The computed regression lines are inserted in Figs. 2 and 3.

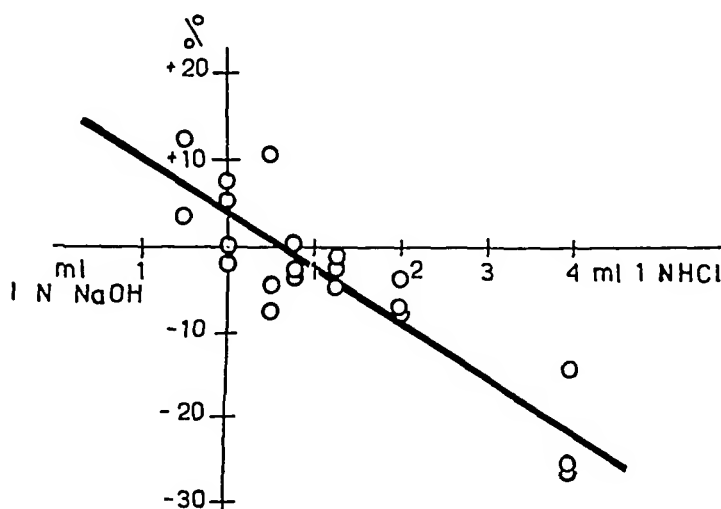


Fig. 2. Change in the CO_2 combining power 5 minutes after injection of dialyzed casein digest. The ordinate indicates the percentage change in the CO_2 combining power, the abscissa shows the amount of 1.00 N NaOH (to the left of the origo) and of 1.00 N HCl (to the right of the origo) added to 5 ml 25 % Aminosol solution. The inserted line is the computed regression line.

As regards the 5 minutes value, it is found that an addition of 0.62 ml 1 N HCl has no effect on the CO_2 combining power. The corresponding value for 30 minutes is 1.63 ml 1.00 N HCl. This signifies that within 5 minutes after the injection an Aminosol solution with a pH-value of 5.1 has no effect on the CO_2 combining power. Within 30 minutes a solution with a pH-value of 4.0 is completely neutralized by the organism.

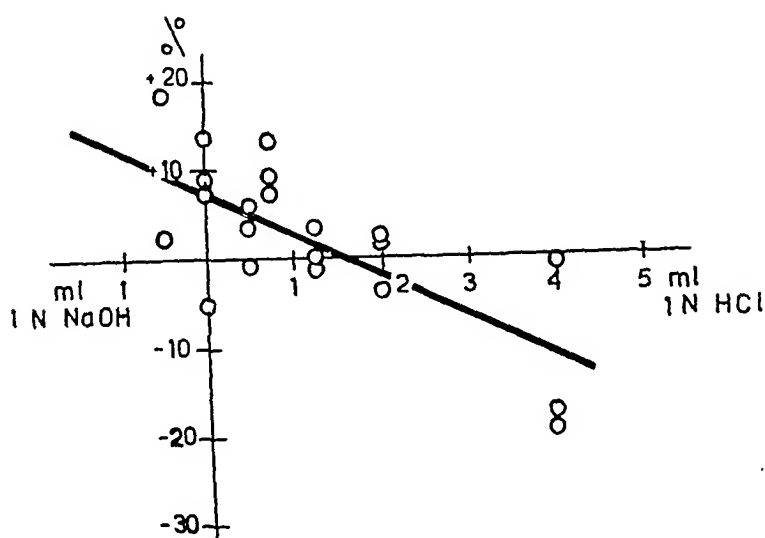


Fig. 3. Change in the CO_2 combining power 30 minutes after injection of dialyzed casein digest. Notations the same as in Fig. 2.

3. Alkali Content in the Ash from Aminosol.

Method. The nitrogen content in the vacuum dried Aminosol was determined according to the method of Kjeldahl (PETERS and VAN SLYKE 1932). The incineration of the Aminosol was made in a platinum crucible in an electric furnace (ca. 550°C). When the ash was quite white, it was weighed, whereupon it was dissolved in distilled water and mixed with a certain surplus of 0.100 N HCl. The solution was boiled and titrated with 0.100 N NaOH. As an indicator, phenol red was used. From the values thus obtained, the amount of 0.100 N HCl required to neutralize the ash from 1 g Aminosol was then computed. The results of these tests are given in Table II. This investigation thus shows the amount of fixed alkali in the Aminosol.

Table II.

Nitrogen %	13.1
Ash %	6.3
ml 0.100 N HCl required for neutralization of ash from 1 g Aminosol	3.88

From these values it can be estimated that the amount of 1.00 N HCl required for neutralization of the ash from 5 ml 25 % Aminosol is 0.49 ml.

Discussion.

By this investigation it is clearly shown that a change of the pH-value in the intravenously injected casein hydrolyzates here employed affects the carbon dioxide combining power. This action is indeed dependent on the pH-value of the solutions, but, in consequence of the varying degree of buffer at different pH-values, the change in the CO_2 combining power is directly proportional to the amount of acid or base required in order to shift the pH-value of the solution from neutral. Theoretically, one might be tempted to suppose that a casein hydrolyzate with a pH-value of 7.4—7.3 would cause the least change in the CO_2 combining power. The observations made in this investigation show, however, that a neutral amino acid solution tends to raise the CO_2 combining power. This corresponds also with the observations made by LIDSTRÖM in connection with intravenous injection of Aminosol in human subjects. It was in fact found that, if the injected solution had a pH-value of ca. 7.3, the result in several cases was a slight alkalosis, which was scarcely explicable otherwise than as due to the administration of Aminosol.

The reason why a neutral solution of casein hydrolyzate entails a rise in the CO_2 combining power may be that the amino acids are rapidly absorbed in the organism and are partly synthetized to protein. That the amino acids on intravenous injection are rapidly eliminated from the blood stream and stored intracellularly in the tissues is well known (VAN SLYKE and MEYER, 1913). In view of this binding of the amino acids, it may be presumed that a certain amount of alkali is liberated, thus entailing a rise of the CO_2 combining power. By the above-reported ash analysis it has been shown that the amount of fixed alkali that can be liberated corresponds to 0.49 ml of 1.00 N HCl, which is nearly equivalent to the amount of acid (0.61 ml) that can be added to the casein digest without the changes in the CO_2 combining power becoming negative in the 5 minutes' test.

That this partly explains the changes in the CO_2 combining power is probable. It is, however, impossible to explain in this way, the whole tendency to alkalosis in the amino acid solutions, as manifested in the 30 minutes values. The explanation of this is probably to be sought in the metabolism of the amino acids.

The investigation shows that an amino acid mixture (enzymatic casein digest) for intravenous use should have a weakly acid reaction. The most suitable pH-value may be considered to lie between 5.1 and 4.0.

A very considerable advantage of causing the amino acid solutions to have an acid reaction is that the growth of bacteria is partly prevented. In the case of a neutral reaction, on the other hand, the amino acid solutions are an excellent culture-medium for bacteria.

No drawbacks seem to result from the use of amino acid solutions with an acid reaction for subcutaneous injection. Thus, about 100 subcutaneous injections were administered to infants (weighing 2—4 kg) with an Aminosol solution of pH 5.3 —5.1. The dosage was 30 ml of a 3.3 % solution per kg of the body-weight. No reactions in the form of pain or skin changes occurred.

Summary.

The changes of the carbon dioxide combining power in rabbits after injection of a dialyzed enzymatic casein hydrolyzate (Aminosol) were studied, and the following results were obtained.

Injection of a neutral solution of casein digest causes a slight alkalosis.

At a pH-value of 5.1 no change in the CO_2 combining power can be observed in blood samples taken 5 minutes after the injection.

An amino acid solution with a pH-value of 4 causes a fall of the CO_2 combining power 5 minutes after the injection. Thirty minutes after the injection this fall is completely neutralized.

The pH-value of casein digest for intravenous use should lie between pH 5.1 and 4.0.

The above-stated pH-values are applicable only to casein digest with a buffer curve of the type here described. The degree of buffer action in this case is comparatively small between pH 8 and 4.

These results indicate that products formed in the metabolism of intravenously injected casein digest tend to induce a slight alkalosis.

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From the Laboratory for the Theory of Gymnastics, University of
Copenhagen.

The Effect of Alcohol and Some Drugs on the Capacity for Work.

By

ERLING ASMUSSEN and OVE BØJE.

Received 10 November 1947.

It was the purpose of the present experiments to study the effect of alcohol and of caffeine, cocaine, strychnine and nitroglycerin on the ability to perform maximal muscular work in healthy, not exhausted athletes. The substances mentioned have well defined pharmacological effects on the resting organism because of which they have been used commonly as "doping" in different kinds of professional competitive sports. As much of the effect of "doping" may be due to the psychological rather than to the physiological effects of the substances and as laboratory experiments are very scanty or completely lacking (for references up to 1939 see BØJE 1939) it was assumed to be useful to study under controlled conditions the effect of the above five commonly used drugs on the muscle power of normal young athletes.

Methods and Procedure.

The work was performed on a stationary, braked bicycle ergometer. Two grades of work were investigated, one consisting in turning the pedals of the bicycle 35 revolutions, making a total amount of work of 956 mkg, and one in 450 revolutions, making a total work of 9,860 mkg (comp. ASMUSSEN and BØJE 1945). The first kind of work could be performed in 12 to 15 sec. and simulates a 100 m dash in its effect on the organism, whereas the latter, lasting about 5 min, simulates a 1,500 m run.

The ethyl alcohol was given as a solution of from 25 to 75 g of pure alcohol in water 30 to 45 minutes previous to the work. Immediately

Table 1.

Performance Time for 35 Revolutions (956 mkg).

Subjects	Alcohol concentration in blood			
	0	0.1—0.3 ‰	0.3—0.6 ‰	0.6—1.0 ‰
A	13.6 sec	13.4 sec	13.8 sec	13.9 sec
E	13.4 —	—	13.8 —	13.3 —
H	11.8 —	12.0 —	11.9 —	12.2 —
L	13.2 —	13.2 —	13.6 —	13.1 —
mean	13.0 —	(12.8) —	13.3 —	13.3 —

Table 2.

Performance Time for 450 Revolutions (9,860 mkg).

Subjects	Alcohol concentration in blood			
	0	0.1—0.3 ‰	0.3—0.6 ‰	0.6—1.0 ‰
A	5.05 min	5.05 min	5.08 min	5.49 min
E	4.85 —	—	—	4.95 —
H	4.78 —	4.78 —	4.78 —	4.88 —
L	4.84 —	4.80 —	—	4.84 —
mean	4.89 —	(4.88) —	(4.97) —	5.04 —

before start a blood sample was collected from a stab in the finger, and the blood alcohol promille was afterwards determined by the method of WIDMARK (1933).

In the experiments with caffeine, cocaine and strychnine the subjects received pills on alternating days containing one of the mentioned pharmaca, or placebos of similar appearance. The subjects were told to swallow the pills whole and so were unable to judge what kind of pills they had received. After the first few experiments the doses were fixed at 30 cg of caffeine, 8 mg of strychnine or 12 cg of cocaine. The drugs were ingested at such times before the work that the maximal effect should be present during work, viz. the caffeine about 30 min., the strychnine about 1 hour and the cocaine about 15 minutes before the start. The placebos were given at corresponding times.

The nitroglycerin was the only drug that had a subjectively noticeable effect on the subjects. They experienced a feeling of warmth and developed a sudden headache 3 to 5 minutes after swallowing 1 mg of nitroglycerin. Any attempt to deceive the subjects in these experiments was, therefore, useless, and in the nitroglycerin tests, which were made last in the series of experiments, no placebos were given in the controls.

As subjects served healthy male students of physical education. The experiments were carried out with daily trials, alternating between the short and long work to avoid chronic fatigue and with the controls

Table 3.

Performance Time for 35 Revolutions (956 mkg).

Subject	Caffeine		Cocaine		Strychnine		Nitroglycerin	
	0	30 cg	0	12 cg	0	8 mg	0	1 mg
K	15.4 sec	15.2 sec	15.2 sec	15.5 sec	15.4 sec	15.5 sec	14.7 sec	15.1 sec
L	16.4 —	16.4 —	16.6 —	16.1 —	16.4 —	16.2 —	16.1 —	16.0 —
S	13.5 —	13.2 —	13.4 —	13.2 —	13.5 —	13.4 —	12.8 —	12.8 —
mean ...	15.1 —	14.9 —	15.1 —	14.9 —	15.1 —	15.0 —	14.5 —	14.6 —

Table 4.

Performance Time for 450 Revolutions (9,860 mkg).

Subject	Caffeine		Cocaine		Strychnine		Nitroglycerin	
	0	30 cg	0	12 cg	0	8 mg	0	1 mg
K	5.25 min	5.09 min	5.25 min	5.31 min	5.23 min	5.20 min	5.09 min	5.07 min
L	5.16 —	5.05 —	5.16 —	4.99 —	5.15 —	5.18 —	5.01 —	5.03 —
S	5.07 —	5.04 —	5.07 —	5.10 —	5.05 —	5.00 —	4.91 —	4.88 —
mean ...	5.16 —	5.06 —	5.16 —	5.13 —	5.14 —	5.13 —	5.00 —	4.99 —

regularly interspersed. The alcohol experiments lasted from February 1946 to July 1946, the experiments with the other drugs from October 1946 to May 1947.

Results.

The results from the alcohol experiments on 4 subjects are presented in table 1 and 2. The whole material has been divided in classes of increasing alcohol content in the blood and averaged for each subject as listed in the tables. A mean value for all the subjects has been calculated and is listed at the bottom of each table. The number of single determination in each class varies from class to class and from subject to subject so that in the mean values at the bottom of the tables from 5 to 50 single determinations are averaged. In the cases where a class is completely missing in one or two subjects, the mean value is placed in brackets.

The tables show, that low blood alcohol promilles have no effect on the performance time and that the higher concentrations (up to 1.0 ‰) has a slightly deteriorating effect, especially in the long work.

The results from the experiments on 3 subjects with the four drugs investigated are tabulated in tables 3 and 4. The individual results are averages of at least five experiments, with the exception of the nitroglycerin experiments of which only three tests were made on each subject with the two grades of work, owing to the unpleasant effects of the drug. The individual values as well as the mean values listed at the bottom of the tables, show that none of the four substances investigated had any clear cut effect on the capacity for this kind of work. Caffeine alone seems to improve the performance time slightly in the long work.

Discussion.

The negative results of the experiments described above show that the maximal performance of rested, healthy subjects cannot be improved artificially by alcohol or drugs.

It was rather surprising that the well known effects of such drugs as cocaine and caffeine were not noticed by our subjects even though the doses were large. Their failure in noticeably increasing the muscular performances of the subjects are in contrast to the finding of Mosso (1891) and of GRAAF (1939) and to the numerous reports from South America on the effect of chewing coca leaves during exhausting work. The explanation is probably the same, viz. that our subjects were normal and not exhausted. The limit to which will power can force a normal man cannot artificially be pushed further, even by these strong drugs. In long lasting efforts where considerable fatigue sets in it is well possible that cocaine or caffeine (as reported by Mosso, GRAAF a. o.) may stimulate the organism to overcome the inhibitory effects of fatigue. The slight effect of caffeine in our long work points in the same direction.

Strychnine was tested because of its well known effects on the synaptic delay, but as seen from the tables had no effect in this kind of work. The same holds true of nitroglycerin, a drug not unfrequently used by professional marathon bicyclists because of its effect on the coronary vessels. But besides its unpleasantness for the subjects it had just as little effect on the muscular performances as the other drugs.

It seems justifiable, therefore, to conclude from the present experiments that alcohol in small doses (blood alcohol up to 0.6 ‰) has no effect, and alcohol in larger doses only a diminishing

effect on the ability to perform maximal muscular work. Caffeine, cocaine, strychnine and nitroglycerin have no effects on the ability to do maximal work in normal non-fatigued subjects with the possible exception of caffeine. The use of these drugs as "doping" in sports and games consequently should be abandoned.

Summary.

Alcohol, caffeine, cocaine, strychnine and nitroglycerin a. o. are used as "doping" in competitive sports. In the present paper the effect of these substances has been tried in controlled work experiments on normal, non-fatigued athletes. It was found that none of them could increase the ability to perform maximal muscular work, lasting about 15. sec or about 5 minutes, with the possible exemption of caffeine in the longer work.

The expenses of this work have been covered by grants from the *Frk. P. A. Brandts Legat* foundation which is gratefully acknowledged.

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On the Formation of Ammonia in the Kidneys During Acidosis.

By

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FITZ and VAN SLYKE (1917) have voiced the opinion that there is a relation between the bicarbonate content of the blood and the formation of ammonia in the kidneys during acidosis, such that the production of ammonia is larger the smaller the concentration of bicarbonate in serum. FØLLING (1927), however, maintains that no such constant relation exists; he finds that the formation of ammonia during an acidosis induced by ammonium chloride reaches its maximum after 3—4 days, at a time when the acidosis is already decreasing. There is a considerably increased excretion of ammonia even after the reaction in the organism has become normal again. The results of FØLLING are in agreement with some previous work of GAMBLE, BLACKFAN and HAMILTON (1925) who likewise maintain that the formation of ammonia does not reach its maximum until after the lapse of several days.

The experiments of the authors mentioned above have been carried out by producing an acidosis which reaches its maximum and then decreases again rather rapidly. Their experiments therefore do not give reliable information about the question whether a larger production of ammonia might not have been obtained by maintaining the acidosis at a certain definite level over a longer period of time.

The purpose of the present work has been to investigate whether the production of ammonia reaches a definite maximum during

an acidosis which is kept at a certain level, or whether it continues to increase. We further wanted to investigate in which way the maximum depends on the degree of acidosis.

Methods.

Determination of Ammonia in urine. We have made use of the method of FOLAN and BELL (1947) according to which the ammonium ion is exchanged with the Na^+ ion in permutite. Various previous modifications of this method have suffered from the defect that the ammonia is not adsorbed quantitatively by the permutite, since a reversible equilibrium is set up between ammonium ions and positive permutite ions.

Our procedure has therefore been to allow the urine to flow down through a column of Na-permutite in a glass tube, thereby obtaining conditions under which the ammonia could be completely adsorbed to the permutite. At the same time we obtained a very efficient elution of nitrogen containing compounds by running distilled water through the column. By this procedure there is no danger of losing finer particles of permutite. We used a glass tube 30 cm in length and 8 mm in diameter. One end was closed with a piece of filter paper, fastened by a piece of rubber tubing. The tube was filled with 2 g of rather fine-grained permutite. A suitable amount of urine, containing ca. 0.1 millimoles of ammonia, was then poured through the column and it was washed through with 2 x 10 ml distilled water. The filter paper was removed and by means of distilled water the permutite was washed down into a 50 ml KJELDAHL tube belonging to a PARNAS-WAGNER distillation apparatus.

It does not affect the analyses if some particles of permutite remain on the filter paper, since they come from the lowest part of the absorption column. The amount of ammonia is determined by an ordinary macro-KJELDAHL determination by distilling the ammonia into a receiver flask containing 5 ml 0.02 N HCl. The titration is carried out with 0.02 N NaOH and with methyl red as an indicator.

Since it was impossible to obtain permutite absolutely free of ammonia a blank was carried out. The values thereby obtained were ca. 0.05 millimoles of ammonia for 2 g of permutite.

The reliability of the method was tested by analysing a solution of the following composition:

100 ml 2 N NH_4Cl + 25 g NaCl + 25 g KCl in 1 liter of water. 10 determinations with permutite on 10 ml of the above solution gave an average value of 0.05 millimoles of ammonia $\pm 0.5\%$. 10 direct KJELDAHL determinations gave an average value of 0.0529 millimoles of ammonia. The ammonia was therefore practically completely adsorbed on the permutite, and the standard deviation in our method of analysis is of the same order of magnitude as in the direct KJELDAHL determination.

If larger quantities of ammonia are present in the solution to be analysed we get complete adsorption up to a certain limit, which is at approximately 0.3 millimoles when 2 g of permutite are used. Beyond this limit a very great decrease in the fraction of ammonia adsorbed by the permutite was observed.

When the analysis is carried out on urine, one source of error is that other nitrogen containing substances than ammonia can be adsorbed by the permutite and given off as ammonia in the KJELDAHL determination. Urea and mono-amino acids are not adsorbed, but di-amino acids are adsorbed. In order to estimate the order of magnitude of the error originating in this way, we analysed the urine from a subject during alkalosis. We found results corresponding to ca. 0.1 millimoles of ammonia in the 1 hour urine. Since the excretion of ammonia during these experiments was between 3 and 10 millimoles per hour, the maximum error caused by these substances is relatively small. Finally, one may well imagine that also during alkalosis small amounts of ammonia are present in the urine so that the actual error is still smaller.

I am much indebted to Dr. JØRGEN MØLLER for having synthesised the permutite and thus having enabled me to carry out these experiments.

The determinations of pH in urine were carried out by means of a glass electrode. The temperature during these measurements was in one experimental period ca. 20° C and in the other 38° C.

The titratable acidity in urine was determined by titration with 0.02 N NaOH from the pH of the urine to $\text{pH} = 7.2$ which corresponds to the pH in serum at the degree of acidosis in question. Before the titration the carbon dioxide was ventilated from the urine after addition of HCl, and a glass electrode was used for the control of pH.

The total carbon dioxide in serum was determined according to VAN SLYKE and NEILL (1932).

Experimental Procedure.

Acidosis was induced by means of ammonium chloride except for a single experiment on a dog in which HCl was used. It turned out to be necessary to give ca. 0.8 millimoles of ammonium chloride per kg body weight in order to get a decrease of 1 milliequivalent (m.e.) per liter in the total serum carbon dioxide. To maintain this degree of acidosis it was necessary to give every 24 hour an amount of ammonium chloride somewhat higher than that corresponding to the formation of ammonia in the kidneys.

To induce the acidosis we used a 10 % Ammonium chloride solution flavoured with 30 % sugar, and for its maintenance we used ammonium chloride tablets a 40 cg.

For determination of the degree of acidosis various methods were considered. It turned out to be extremely difficult to determine pH directly in the blood by means of a glass electrode with an accuracy larger than 0.05 pH units, since the asymmetric potential of the elec-

trode, which had otherwise been constant for days, showed variations of this order of magnitude on contact with the blood. However, it was considered unnecessary to obtain an absolute measure of the degree of acidosis. We were content to have a relative measure, capable of showing the variations in the degree of acidosis and we therefore chose to determine the total carbon dioxide in serum of venous blood. This quantity was fairly constant in samples drawn at short intervals (see Table 1.).

Table 1.

Determination of total carbon dioxide in serum of blood from v. cubiti in male subject J. V. H.

Date	Time of day	Total CO ₂ in serum in millimoles per liter
11/.....	16.00	18.2
	16.30	18.7
	17.00	18.8
12/.....	18.00	15.4
	18.30	15.0
	19.00	15.0

Since we were primarily interested in obtaining a relative measure of the degree of acidosis we did not see any advantage in determining total carbon dioxide in venous blood which had been "arterialized" by immersing the arm in hot water. By this procedure variations in the content of carbon dioxide might easily arise in consequence of a changing respiration, and we would further lose the advantage of the fact that the tissues in the arm act as buffers for rapid variations in the content of carbon dioxide.

Moreover it appeared that by calculating the hydrogen ion concentration on the basis of the determination of the carbon dioxide tension in alveolar air and the content of bicarbonate in serum from blood arterialized in the way just mentioned, we obtained values which during acidosis were more than 0.1 pH units higher than those obtained by a direct measurement of pH by means of the glass electrode in the same sample of blood. During alkalosis the results of both methods were more in accordance with one another. The reason for this is probably that the blood does not become completely arterialized, and this fact may particularly influence the calculation of the hydrogen ion concentration when the content of bicarbonate is small.

The hydrogen ion concentration and the content of ammonia were as a rule determined in 24 hour samples which had been stored at a temperature less than 10° C; some drops of toluene had been added for the purpose of preservation. Any precipitate, if present, was uniformly

distributed in the liquid by thorough shaking before samples for the ammonia determination were drawn. Before the determination of pH the precipitate was dissolved by a slight heating of the sample.

Since in all cases pH has been less than 5.5 it was found unnecessary to store the samples under paraffin oil.

Experimental Results.

Table 2 gives the results of an experiment in which a weak acidosis has been induced and maintained. The content of bicarbonate in serum was between 17 and 20 m.e. per liter. The formation of ammonia slowly increased until the fourth day of the experiment, and then remained very constant. The pH in the urine

Table 2.

Acidosis induced by means of ammonium chloride in male subject J. V. H.

Weight 53 kg, urea clearance 71 ml per minute.

Period of collecting urine	NH ₃ in urine (in millimoles per 24 hours)	pH in urine at 38°	Diuresis (in ml per 24 hours)	Date	Total CO ₂ in serum in millimoles per liter (at about 12.00)	Ingestion of NH ₄ Cl (in millimoles per 24 hours)
24/1 12.00—25/1 8.00..	103	4.81	2250	24/1		350
25/1 8.00—26/1 ..	141	5.03	1720	25/1	19.3	250
26/1 —27/1 ..	168	5.09	1130	26/1	18.0	200
27/1 —28/1 ..	203	5.19	1360	27/1	20.4	350
28/1 —29/1 ..	215	5.28	1450	28/1	18.7	300
29/1 —30/1 ..	213	5.24	1390	29/1	17.8	280
30/1 —31/1 ..	231	5.23	1470	30/1	19.1	280
31/1 —1/2 ..	235	5.19	1740	31/1	18.3	280
1/2 —2/2 ..	209	5.39	1240	1/2		280
2/2 —3/2 ..	233	5.23	1390	2/2	19.9	350
3/2 —4/2 ..	222	5.27	1260	3/2		300
4/2 —5/2 ..	205	5.38	1040	4/2	17.5	280
5/2 —6/2 ..	209	5.40	1150	5/2		280
6/2 —7/2 ..	216	5.37	1290	6/2	18.1	280
7/2 —8/2 ..	225	5.29	1370	7/2		280
8/2 —9/2 ..	222	5.33	1110	8/2	19.7	280

On 2/2 (between 8.00 and 12.00) 35 g of sodium bicarbonate were ingested. Total carbon dioxide in serum was at 15.00: 32.5 millimoles per liter.

Period of collecting urine	Diuresis in ml per hour	NH ₃ in millimoles per hour	pH
15.00—16.00	40	6.75	5.41
16.00—17.00	37.5	5.60	5.49

Table 3.

Acidosis induced by means of ammonium chloride in the same subject as in Table 2.

Period of collecting urine	NH ₃ in urine (in milli-moles per 24 hours)	pH in urine at 38° C	Titra-table acidity in urine (in m.e. per 24 hours)	Diuresis (in ml per 24 hours)	Date	Total CO ₂ in serum (milli-moles per liter)	Inges-tion of NH ₄ Cl (in milli-moles per 24 hours)
¹⁵ / ₁₀ 15.00— ¹⁶ / ₁₀ 8.00	126	4.73	55	2020	¹⁵ / ₁₀	14.9	590
¹⁶ / ₁₀ 8.00— ¹⁷ / ₁₀ 8.00	147	4.93	48	1440	¹⁶ / ₁₀	14.0	200
¹⁷ / ₁₀ — ¹⁸ / ₁₀	183	5.06	46	1120	¹⁷ / ₁₀	16.3	300
¹⁸ / ₁₀ — ¹⁹ / ₁₀	217	5.27	41	1190	¹⁸ / ₁₀	14.0	300
¹⁹ / ₁₀ — ²⁰ / ₁₀	226	5.24	38	1250	¹⁹ / ₁₀	14.5	300
²⁰ / ₁₀ — ²¹ / ₁₀	210	5.31	37	1070	²⁰ / ₁₀	15.8	300
²¹ / ₁₀ — ²² / ₁₀	230	5.25	42	1310	²¹ / ₁₀	14.9	300

was lowest on the first day and then increased with increasing formation of ammonia.

Similar results were obtained in an analogous experiment on another subject.

After termination of the experiment the subject was given 35 g of sodium bicarbonate. The serum bicarbonate then increased to 32.5 m.e. per liter, which is a rather high value. In spite of this the pH of urine was still small and the formation of ammonia high, even higher than during the initial stage of the acidosis.

Table 3 shows the results of an experiment on the same subject, during which a more pronounced acidosis has been induced. In order not to endanger the health of the subject the duration of the experiment was restricted to 7 days. The content of bicarbonate in serum was between 14 and 16.3 millimoles per liter. Also in the present case the excretion of ammonia reached its maximum on the fourth day, and during the following days it remained fairly constant at about the same level as in the experiment described in Table 2. The variations in pH were likewise in good agreement with those in Table 2.

The daily excretion of acid, determined by titration to pH 7.20, was very much smaller than the excretion of ammonia. It assumed its highest values during the first days of the experiment and did not increase during the acidosis.

Strangely enough the amount of NH₄⁺ given pr. os. exceeds the total amount of NH₄⁺ and titratable acid excreted with the urine. This was not to be expected, since normally a surplus of

Table 4.

Formation of ammonia in dog during acidosis; weight 11.5 kg., urea clearance 18 ml per minute.

Period of collecting urine	Diuresis per hour	Ammonia (in millimoles per hour)	Date	Total CO ₂ in serum (in millimoles per liter)	Ingestion of HCl (in millimoles per 24 hours)
¹⁴ / ₃ 7.40—8.53..	49.5	0.97	¹⁴ / ₃	16 (at 10.00)	100 (between 6.50 and 7.55)
8.53—9.34..	95	2.15			60 (after 13.00)
9.34—10.13..	172	2.54			
10.13—10.56..	232	2.62			
10.56—11.46..	218	2.55			
11.46—12.34..	190	2.43			
¹⁵ / ₃ 12.25—13.26..	183	2.67	¹⁵ / ₃	14	50
¹⁶ / ₃ 15.30—16.25..	195	2.42	¹⁶ / ₃	18	70
¹⁷ / ₃ 12.10—13.13..	175	2.83	¹⁷ / ₃	15	60
¹⁸ / ₃ 12.20—13.18..	191	2.35	¹⁸ / ₃	14	60
¹⁹ / ₃ 12.30—13.40..	227	2.64	¹⁹ / ₃	17	

acid is produced in the organism. It is not possible for me to give a satisfactory explanation to this as I have not controlled the food eaten during the experimental periods, nor have I investigated if any NH₄Cl was excreted with the feces.

Table 4 shows the formation of ammonia in a dog during acidosis induced by ingestion of HCl. It reaches its maximum after a couple of hours and then remains constant during the following days. In control experiments it was shown that the ammonia formation behaves similarly during acidosis induced by the administration of ammonium chloride.

Discussion.

The experiments show clearly that the formation of ammonia in human subjects during acidosis exhibits a certain inertia, so that it reaches its maximum intensity after some days but continues some time after cessation of the acidosis. This confirms the results of GAMBLE, BLACKFAN and HAMILTON and of FØLLING, but disagrees with those of FITZ and VAN SLYKE. In dogs the formation of ammonia reaches its full magnitude in a few hours.

The experiments further show that the maximum rate of ammonia formation reached during an acidosis is rather well defined and independent of the degree of acidosis. It may be worth pointing out that this maximum seems to be approximately the same

man as in the dog, when calculated per ml urea clearance. In the subject E. V. C. the quantity

$$\frac{\text{Maximum rate of ammonia formation}}{\text{urea clearance}}$$

is ca. $220/71 = 3.1$. In another male subject the same quantity is ca. $230/68 = 3.4$. In two dogs we found the values $65/18 = 3.6$ resp. $50/11.5 = 3.5$.

The excretion of acid in the urine, measured by titration to pH 7.20, did not in general constitute as great a part of the neutrality regulation as the formation of ammonia. This is due to the fact that the excretion of acid is limited by the buffer capacity of the urine, since the hydrogen ion concentration cannot exceed a certain definite level. If the buffer capacity is increased, the excretion of acid can increase and may far exceed the formation of ammonia. By intravenous administration of phosphate to a dog during an acidosis PITTS and ALEXANDER (1945) obtained a rise in the titratable acid to more than 30 m.e. per hour. In a similar experiment carried out by me the titratable acid reached 33 m.e. while no change in the production of ammonia was found.

The excretion of acid was largest during the first days of the experiment, before the formation of ammonia had reached its maximum. However, the difference was so small that we cannot regard it as a compensation for the reduced formation of ammonia.

The problem of the variations in hydrogen ion concentration will be further discussed in a later paper.

Summary.

A description is given of a modification of the method of FOLIN and BELL for the determination of ammonia in urine by means of permutite.

It is demonstrated that both in man and dog the formation of ammonia during acidosis reaches a rather well defined maximum independent of the degree and duration of the acidosis. In man this maximum is reached after about 4 days, and the formation of ammonia continues at a high rate some time after cessation of the acidosis.

In dogs the formation of ammonia attains its maximum in the course of a few hours.

The pH in human urine is lowest in the initial stage of the acidosis and increases with increasing formation of ammonia.

I am indebted to P. Carl Petersens Fond for support of this work.

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Some Investigations on the Carbon Dioxide Tension of the Urine in Man.

By

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The present work is an attempt at an investigation of the laws that govern the carbon dioxide tension in urine, in particular its dependence on the pH and buffer capacity of the urine and on the diuresis.

STRASSBURG (1872), from an investigation of 3 cases, found that the carbon dioxide tension in urine was considerably higher than that of venous blood.

SENDROY, SEELIG and VAN SLYKE (1934) found that the carbon dioxide tension in urine varies between 40 and 200 mm and does not depend on the pH of the urine. SARRE (1938) has found similar results but did not, however, find values exceeding 100 mm. This author points out the possibility that the hydrogen ion concentration in the urine may change so much during the time needed for its collection (about half an hour) that the values actually measured do not correspond to the original tensions.

SARRE has thereby drawn attention to an important fact, which previously by MAINZER and BRUHN (1931) had been made responsible for the large variations in the carbon dioxide tension in urine. However, neither SARRE, nor MAINZER-BRUHN nor the other workers have got this factor under control, since they have taken no precautions to keep the hydrogen ion concentration constant during the experiments. In particular in the experiments of SENDROY, SEELIG and VAN SLYKE there has been a danger of variations in pH, since their experimental subjects have carried out muscular exercise or have been given bicarbonate.

In some recent investigations concerning the excretion of bicarbonate in the urine PITTS and LOTSPEICH (1946) give some estimates of the p_{CO_2} in urine of dogs under different conditions. In their experiments pH has been relatively constant and the diuresis very large and so the error originating from mixture of different portions of urine must be considered quite small. They find the p_{CO_2} in urine with $\text{pH} < 6.0$ approximately to equal the tension in arterial blood, but largely to exceed it when $\text{pH} > 7.0$, the difference in tension in many cases being more than 50 mm. The authors calculate p_{CO_2} in urine and arterial blood from the content of total carbon dioxide and pH, using a pK value of 6.1. pH has been measured at room temperature and corrected to 37°C. by subtracting 0.01 pH unit per degree difference in temperature. As the authors state themselves, this is a rather inaccurate method. pK cannot be expected to be constant in different samples of urine (cf. SENDROY et al., 1934) and an error of 0.1 in pK or pH will result in an error of 26 % in the calculated tension.

The purpose of the investigations here presented has been to measure directly with tonometer the carbon dioxide tension of the urine of man under various conditions, but always subject to the condition that the hydrogen ion concentration has been kept as constant as possible during one and the same experimental period.

Methods of Analysis.

For determinations of pH we used the glass electrode. The voltage was measured by means of a thermionic tube potentiometer.

The urine was kept under paraffin oil and at a temperature of $20^\circ \pm 2^\circ\text{C}$.

As standard buffer a solution was used consisting of equal amounts of 0.1 n acetic acid and 0.1 n sodium acetate ($\text{pH} = 4.625$ at 20°C).

For determinations of the carbon dioxide tension in urine we have used a tonometer similar to that of SENDROY, SEELIG and VAN SLYKE (1934). Except for the volume this tonometer corresponds completely to that described by VAN SLYKE, SENDROY and LIU (1932) for the determination of tensions in blood.

Since we were unable to procure a four-way stopcock, which was necessary for this apparatus, I am indebted to EINAR LUNDSGAARD for having constructed another tonometer using a three-way stopcock. However, the analytical procedure is about the same in both cases. The volume of our tonometer was 21.2 ml instead of 50 ml in the apparatus of SENDROY, SEELIG and VAN SLYKE (1934). This choice of dimen-

sions enabled us to measure the tensions in smaller samples of urine. However, at the same time we increase the error that originates from the circumstance that p_{CO_2} in the CO_2 -mixture used in the tonometer bubble is different from p_{CO_2} in the urine sample. If the sample is so acid as to contain practically no bicarbonate, this error γ will be given to a high degree of approximation by the expression

$$\gamma = (p_2, \text{CO}_2 \div p_1, \text{CO}_2) \times \frac{p_2, \text{CO}_2 \times 0.95}{p_2, \text{CO}_2 \times 20.25 \times 0.522}$$

where p_1, CO_2 and p_2, CO_2 are the carbon dioxide tensions in urine sample and tonometer bubble respectively; 0.95 is the volume of the tonometer bubble, 20.25 the volume of the urine in the tonometer, both quantities being expressed in ml; 0.522 is the average absorption coefficient of CO_2 in urine at 38°C (SENDROY, SEELIG and VAN SLYKE, 1934). We have approximately

$$\gamma = 0.08 (p_2 - p_1).$$

Since the difference between p_1, CO_2 and p_2, CO_2 in all experiments at pH around 5 has been less than 5 mm we get $\gamma < 0.4$ mm. At higher values of pH, where appreciable amounts of HCO_3^- are present, γ is smaller; γ is the smaller the higher are the values of pH and the buffer capacity of the urine.

In the experiments to be reported below the difference ($p_1 - p_2$) has always been less than 5 mm at a pH around 5, and less than 10 mm at higher values of pH. Thus, in acid samples of urine γ has been less than 0.4 mm, and less than 0.8 mm in the samples with pH > 6.0 . Since the error in the tension measurements is somewhat higher than these figures, we have not taken any correction into account.

On introduction into the tonometer the sample of urine will take up resp. give off carbon dioxide, according to whether p_1 is lower or higher than p_2 . At the same time p_2 will decrease since the CO_2 -bubble is "diluted" by taking up water vapour. (In my experiments I have used a dry CO_2 -mixture in the tonometer bubble.) Furthermore p_2 will increase on heating the tonometer to 38° , but the error arising from this circumstance will be partly eliminated in the subsequent equilibration to atmospheric pressure.

These errors are, however, of no practical importance, since none of them are larger than 0.1 mm, and the two last mentioned errors tend to cancel each other.

In order to test the accuracy of the method we have measured p_{CO_2} in an aqueous solution of CO_2 at pH = ca. 3 and compared it to the tension calculated from the volume of CO_2 found by liberating the gas into a partial vacuum. By this double determination of p_{CO_2} on 10 different solutions the following results were obtained:

Co ₂ solution ...	1	2	3	4	5	6	7	8	9	10
pCO ₂ "directly"										
determined..	49.4	48.7	50.5	52.7	54.1	53.5	48.1	49.2	55.1	51.5
pCO ₂ determined										
with tonometer	47.3	49.1	50.2	47.5	55.5	54.0	47.1	47.3	52.8	50.8
Difference	-2.1	+0.4	-0.3	-5.2	+1.4	+0.5	-1.0	-1.9	-2.3	-0.7
Average difference: —1.1										

pCO₂ in the tonometer bubble was in all determinations 50 mm. It seems, therefore, that tonometer determinations give somewhat lower values than direct determinations.

We have further assured ourselves that in the tonometer determinations minute quantities of urine do not pass over into the VAN SLYKE apparatus by adhering to the mercury. Considerable errors might arise in this way in urines with a high content of HCO₃⁻. We measured pCO₂ in the ordinary way in a solution of carbonate containing ca 300 milliequivalents CO₃⁻ per liter corresponding to the maximum content of HCO₃⁻ to be expected in urine, and found pCO₂ < 1 mm.

The determination of the carbon dioxide tension in alveolar air was carried out in the following way: in immediate continuation of a normal expiration a maximum expiration was made through a long rubber tubing ending under water. Samples for analysis were drawn from the air enclosed in the tubing. The content of carbon dioxide, expressed in per cent, was determined according to VAN SLYKE, SENDROY and LIU (1932).

The content of total-CO₂ in serum and urine was determined according to PETERS and VAN SLYKE (1932), and the content of HCO₃⁻ in urine by subtracting the carbon dioxide physically bound from the total CO₂.

The titration curve of urine after removal of CO₂ by ventilation (at a pH between 4—5) was determined by titrating with 0.01 n NaOH using a glass electrode to give the values of pH.

Experimental Conditions.

The experiments reported in the present paper have all been carried out on one and the same experimental subject, a 20 years old man of weight 55 kg. However, on other young men certain control experiments have been carried out, indicating that the results obtained are not characteristic of the individual experimental subject.

Experiments have been carried out both during acidosis and alkalosis and at neutral reaction.

In acidosis it is easy to keep the hydrogen ion concentration in the urine constant. Moreover, in acid urine changes in pH will cause only insignificant changes in pCO₂, since only a small fraction of the total CO₂ is present as HCO₃⁻.

During alkalosis the pH of the urine will likewise be fairly constant, provided only that the diuresis is approximately constant. Mixing of two samples of urine within a period will entail only a minor change in p_{CO_2} , since the buffer capacity of urine in this interval is very small, in particular when, on account of the alkalosis, only insignificant quantities of ammonia are present.

The greatest difficulties are encountered when pH is between 5.5 and 7.0, since in this interval the reaction is fluctuating. Under these conditions we have succeeded in obtaining a satisfactorily constant pH only when the diuresis is large. However, this latter fact entails the advantage that it is possible to collect urine over shorter intervals of time.

We have always taken several samples of urine in series, in order to make sure that p_{CO_2} was constant under constant conditions. Moreover the hydrogen ion concentration was measured in samples drawn both before and after those used for the determination of p_{CO_2} .

The day before an experiment the subject received a diet poor in calcium, in order to avoid precipitation of calcium phosphates in the urine at alkaline reaction. All the experiments were carried out in the morning before the subject took any food.

Experimental Results.

p_{CO_2} in urine with $pH < 5.5$.

Table 1 shows the results from 3 days after administration of ammonium chloride. On the first day the subject shows a normal content of bicarbonate in serum, on the other two days there is a decrease in the content of bicarbonate and at the same time a decrease in the carbon dioxide tension of the alveolar air.

The hydrogen ion concentration in the urine has surprisingly enough its lowest value at the lowest value of the bicarbonate content in serum. This fact will be investigated more closely in a later communication.

It is further seen that p_{CO_2} in the urine is about 3 mm higher than p_{CO_2} in the alveolar air, and that a decrease of the latter tension entails a corresponding decrease of the tension in the urine.

Further experiments under similar conditions gave analogous results. In 30 different samples of urine the carbon dioxide tension was on the average 3.3 ± 2.0 mm higher than the carbon dioxide tension in the alveolar air, determined at the same time.

p_{CO_2} in urine with $pH = \text{ca. } 6.5$.

Table 1.

Time of collecting sample of urine	pH in urine at 20°	PCO ₂ in urine at 38°	PCO ₂ in alveolar air	Total CO ₂ in serum
9—10 h	4.80			26 milliequiv. per liter
10—11	4.78	43 mm	41.9 mm	
11—12	4.77	46	40.9	
12—13	4.80	45	41.6	
13—14	4.79			
9—10 h	5.17			21 milliequiv. per liter
10—11	5.20	42 mm	35.8 mm	
11—12	5.21	37	35.6	
12—13	5.25	37	37.0	
13—14	5.25			
9—10 h	5.30			17 milliequiv. per liter
10—11	5.35	35 mm	31.7 mm	
11—12	5.33	35	32.1	
12—13	5.39	36	31.3	
13—14	5.38			

PCO₂ in urine and alveolar air when pH < 5.50 in the urine. Exp. subj. J. V. H. Diuresis < 100 ml per hour.

Table 2.

Time of collecting sample of urine	pH in urine at 20°	PCO ₂ in urine at 38°	PCO ₂ in alveolar air	Total CO ₂ in serum
10 —10.10 h	6.52		41.9 mm	26 milliequiv. per liter
10.10—10.20	6.54	62 mm		
10.20—10.30	6.54	58		
10.30—10.40	6.54	59		
10.40—10.50	6.55			
10 —10.10 h	6.57		42.8 mm	27 milliequiv. per liter
10.10—10.20	6.55	57 mm		
10.20—10.30	6.58	58		
10.30—10.40	6.60	58		
10.40—10.50	6.62			

PCO₂ in urine and alveolar air when pH = ca 6.50 in the urine. Exp. subj. J. V. H. Diuresis = ca 900 ml per hour.

As mentioned above we have under these conditions only carried out experiments when the diuresis was large.

Table 2 gives the results for two days. The difference between the carbon dioxide tensions in the urine and in the alveolar air is somewhat larger than 15 mm and hence considerably larger

than during acidosis. The results were also here fully reproducible from day to day.

p_{CO_2} in urine with $\text{pH} > 7.0$.

Table 3 gives the results of an experiment during alkalosis induced by administration of 40 g NaHCO_3 before the experiment and then 2 g every hour.

Table 3.

Time of collecting sample of urine	Diuresis in ml per hour	pH in urine at 20°	PCO_2 in urine at 38°	HCO_3^- in urine in m. eq. per liter	HCO_3^- in urine per hour in m. equiv.	PCO_2 in alveolar air in mm.	Total CO_2 in serum per liter in m. equiv.
10.43—11.15		7.98				50.2	
11.15—11.51	221	8.00	111 mm	270	59.7		41
11.51—12.26	143	8.00	119	285	40.8		
12.26—13.18	119	8.01	117	272	32.4		
13.18—14.02	91	8.05	114	300	27.3		
14.02—14.48	57	8.11	109	329	18.8		
14.48—15.30	72	8.09	114	330	23.8		40
15.30—16.25	49	8.10	111	324	15.9		
16.25—16.45	61	8.10				51.6	

PCO_2 in urine and alveolar air when $\text{pH} > 7.00$ in the urine. Exp. subj. J. V.
H. Diuresis small.

P_{CO_2} in the urine is on the average ca. 60 mm larger than p_{CO_2} in the alveolar air. Both the hydrogen ion concentration and the tensions are satisfactorily constant. It is therefore very likely that the carbon dioxide tension in the urine emptied into the bladder really assumed this high value.

In further experiments we investigated the dependence of P_{CO_2} on the magnitude of the diuresis at constant bicarbonate content in the blood. Table 4 shows the results of an experiment in which the diuresis was slightly larger than 800 ml per hour. p_{CO_2} in the urine is here somewhat smaller, on the average ca. 25 mm larger than p_{CO_2} in the alveolar air. At the same time pH has decreased to about 7.40. The concentration of bicarbonate is reduced to ca. $\frac{1}{8}$, but the excretion per hour is of the same order of magnitude as at a smaller diuresis.

In Fig. 1 we have reproduced the results of all the measurements of p_{CO_2} in the urine of our experimental subject during alkalosis. In all cases p_{CO_2} in the alveolar air was between 49 and

Table 4.

Time of collecting sample of urine	Diuresis in ml per hour	pH in urine at 20°	PCO ₂ in urine at 38°	HCO ₃ ⁻ in urine in m. eq. per liter	HCO ₃ ⁻ in urine per hour in m. equiv.	PCO ₂ in alveolar air	Total CO ₂ in serum in m. equiv. per lit.
16.45—17.05	837	7.49					
17.05—17.25	819	7.44	80 mm	39.8	32.6		
17.25—17.48	892	7.43	71	37.5	33.5		
17.48—18.09	855	7.40	72	34.0	29.1	49.5 mm	
18.09—18.30	809	7.42	81	39.1	31.6		40
18.30—18.49	716	7.43					

PCO₂ in urine and alveolar air when pH > 7.00 in the urine. Exp. subj. J. V. H. Diuresis > 800 ml per hour.

52 mm and total CO₂ in serum was between 38 and 41 milliequivalents per liter. We are therefore presumably justified in representing the quantities together.

When the diuresis is less than ca. 250 ml per hour, we find no relation between diuresis and p_{CO₂} in the urine, but at values of the diuresis between 250 and 1,000 ml per hour p_{CO₂} decreases

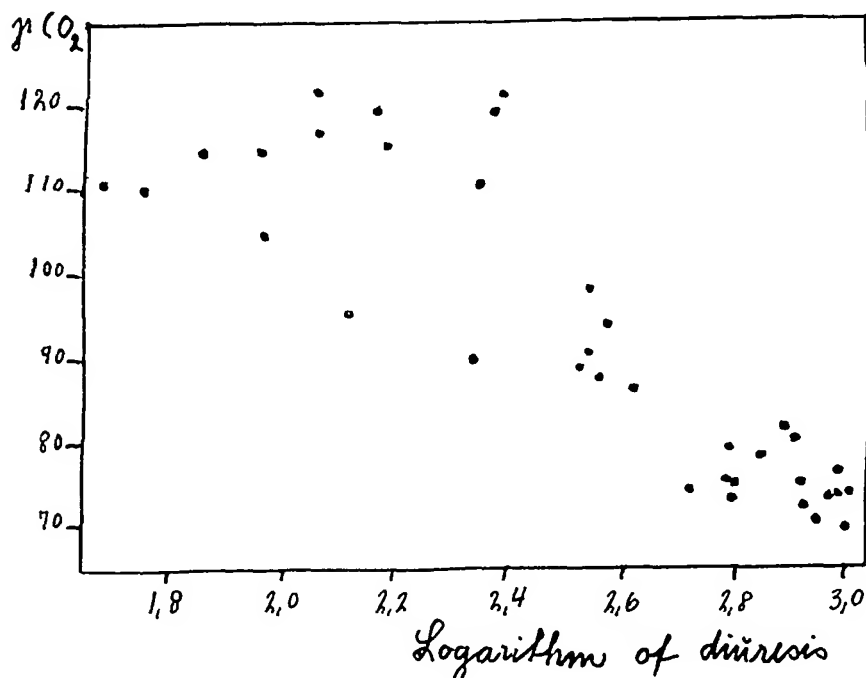


Fig. 1. Relation between logarithm of diuresis and pCO₂ in urine. pH in urine > 7.00.

with increasing diuresis. Further there seems to be an approximately linear relation between carbon dioxide tension and logarithm of diuresis.

In most of our experiments during alkalosis we determined the titration curve of the urine after removal of CO_2 by ventilation. It was namely assumed that the high values of the carbon dioxide tension in the urine might be due to a transformation of HCO_3^- to CO_2 , and by means of the titration curve it is possible to calculate the change in pH due to such a transformation. A further discussion of this point is given below.

Discussion.

1. *The relation between p_{CO_2} and pH in the urine.*

Carbon dioxide is an easily diffusible substance, so the tensions in blood and urine might be expected to be approximately equal.

The observed carbon dioxide tensions at $\text{pH} < 5.5$ are in good agreement with this view. The result that the tensions in urine are 3—4 mm higher than those in the alveolar air is in agreement with the condition that p_{CO_2} in the tubular liquid must be somewhat higher than the tension in the venous blood of the kidney, which again must be higher than the tension in the alveolar air.

As already mentioned previous authors with the exception of PITTS and LOTSPEICH have found much higher carbon dioxide tensions in both acid and alkaline urines, but this can be fully explained by the fact that they have taken no precautions to keep the reaction of the urine constant during their experiments. As a curiosity I may mention that on neglecting this condition I have measured carbon dioxide tensions up to 600 mm.

The higher carbon dioxide tensions found in more alkaline urine are rather surprising. On considering the diffusibility of carbon dioxide, the small width of the tubuli and the great vascularisation of the kidneys, it is difficult to imagine the existence of a carbon dioxide tension in the tubular liquid more than twice as high as that in arterial blood. PITTS and LOTSPEICH (1946) state that "presumably bicarbonate reabsorption is effected indirectly in the distal tubule by the exchange of H^+ ions for Na^+ ions, thereby converting bicarbonate in the tubular urine to car-

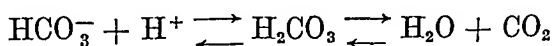
bonic acid" and that "failure to establish equilibrium across the tubular epithelium would account for the fact that the p_{CO_2} of urine is often higher than that of blood, especially when the distal mechanism is loaded and bicarbonate appears in the urine".

This view is supported when we consider the investigations of FAURHOLT (1924) which seem to offer a fuller explanation of the fact that p_{CO_2} is the highest in alkaline urine.

FAURHOLT measured the velocity coefficients at 0°C and at 18°C of the processes that determine the rapidity with which the equilibrium is set up. If we assume that the velocity increases with the same temperature coefficient between 18° and 38°C as between 0° and 18°C , we find that at $\text{pH} = 5$ 50 % equilibrium is reached in 0.1 sec., 90 % in 0.3 sec., and 99 % in 0.6 sec. For values of pH between 5 and 7 the logarithm of the velocity is in rough approximation inversely proportional to pH , and at a pH in the interval between 7 and 8 we find that a 20 % equilibrium is reached in ca. 0.7 sec., 50 % in ca. 2 sec., 90 % in ca. 7 sec. and 99 % in ca. 14 sec.

These figures are of course only approximate, but they show that there is a possibility that the equilibrium between HCO_3^- , CO_2 and pH at alkaline reaction is not set up in the tubuli, but only after the urine has reached the collecting ducts, where conditions are less favourable for diffusion. I would consider this explanation much more probable than the assumption of a carbon dioxide tension in the tubular liquid more than twice as large as that of arterial blood.

Granted the correctness of this assumption the transformation of the filtrate to urine would have to be accompanied by a shift of the process



to the right. Such a displacement may occur on account of an increase either in HCO_3^- or in H^+ . Both these processes are likely to play a rôle.

In the more concentrated samples of urine the content of bicarbonate was higher than 300 milliequivalents per liter, which is a very considerable concentration from the value for serum (ca. 40 m.e. per liter). However, when the diuresis was large the content of bicarbonate was often smaller in urine than in blood during alkalosis. Since the carbon dioxide tension was higher in the urine than in the blood, the hydrogen ion concentration must have

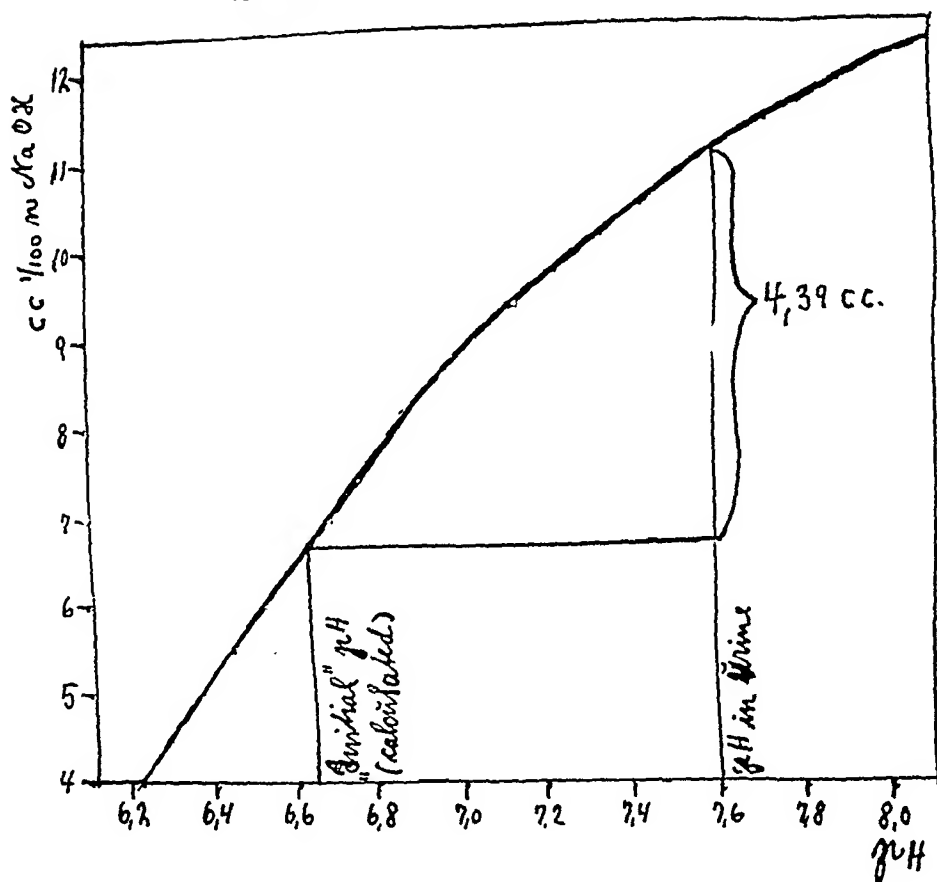


Fig. 2. Example of calculation of "initial" pH in the liquid in the tubule. The figure shows the titration curve of the urine after removal of the CO_2 .

The PCO_2 in the sample in question was 88 mm, the "excess pressure" was 34 mm. The absorption coefficient has been estimated to 0.54. The volume of the titrated sample was 40 mm. 34 mm CO_2 then represents $(0.54 \times 34 \times 40) / (760 \times 22.4) = 0.0439$ m. e. CO_2 which, according to the theory, has been formed by transformation of HCO_3^- . This quantity is equivalent to 4.39 ml 0.01 n NaOH. We determine the point on the curve that corresponds to the pH of the urine (in the present case pH = 7.60). From the ordinate of this point we subtract 4.39, and then determine the point on the curve corresponding to this reduced ordinate. The abscissa of the latter point gives the pH of the urine before the transformation of HCO_3^- to CO_2 had taken place.

been higher in urine than in blood. This might be interpreted to mean that under these conditions, in spite of the alkalosis, the urine has been acidified during its passage through the tubules. In order to investigate whether we really have to reckon with the occurrence of such phenomena, I have calculated the hydrogen ion concentration of the urine before the transformation of HCO_3^- to CO_2 .

For every equivalent of HCO_3^- , which is transformed to CO_2 ,

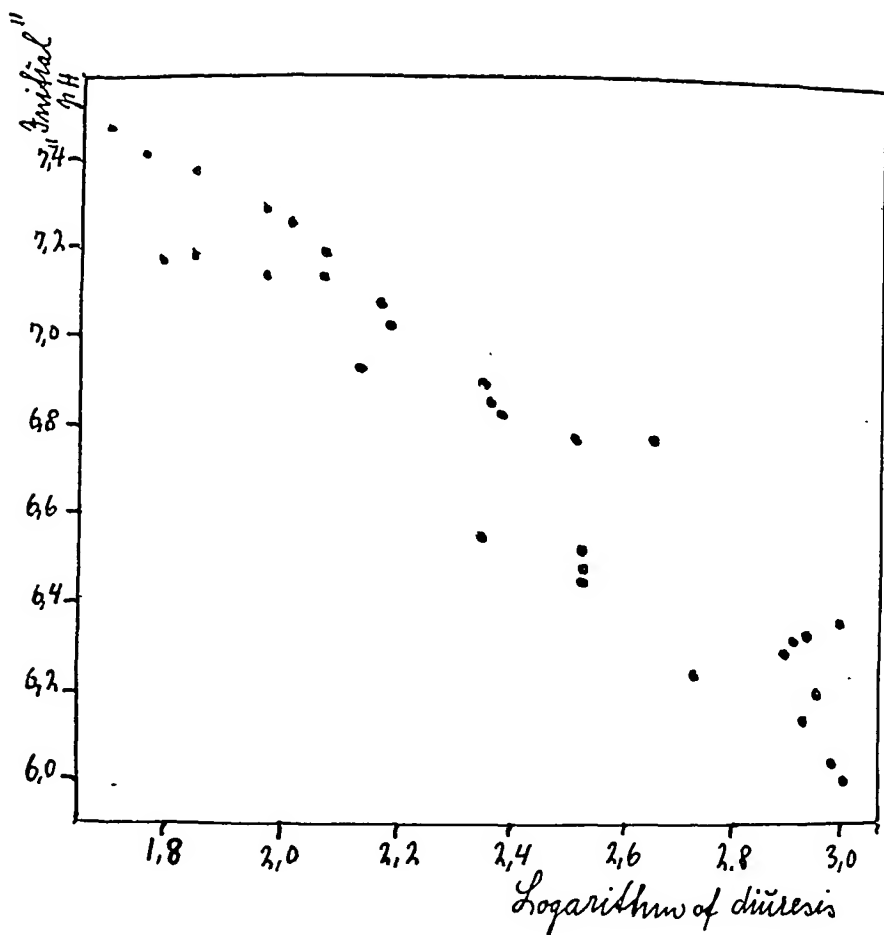


Fig. 3. Relation between logarithm of diuresis and "initial" pH (calculated as shown in fig. 2). pH in urine > 7.00 .

one equivalent of H^+ is consumed. This must be taken from the other buffer systems in the urine, the reaction of which is thereby shifted in alkaline direction. The magnitude of the shift, which occurs when a certain definite amount of H^+ is given off, depends upon the titration curve of the other buffer systems in the urine, and from a knowledge of this curve we are able to calculate the value of pH before the shift.

By way of approximation we assume that the initial p_{CO_2} in the tubular liquid is equal to p_{CO_2} for the alveolar air plus 3.3 mm, corresponding to the conditions during acidosis. This number is subtracted from the p_{CO_2} of the urine, and we compute the corresponding number of milliequivalents of CO_2 (for the absorption

coefficient we have assumed the approximate value 0.51—0.54, according to the concentration of the urine; see SENDROY, SEELIG and VAN SLYKE (1934). The same number of milliequivalents of H^+ have been given off by the other buffer systems in the urine, and in a titration curve, determined for the sample of urine in question after removal of CO_2 by ventilation, we can read off the value of pH before the transformation occurred. Fig. 2 shows by way of example how the calculation is carried out.

Fig. 3 shows the "initial" pH values calculated by the above method for 30 samples of urine during alkalosis. It appears that, granted the correctness of the assumption that the high carbon dioxide tensions are due to a retarded transformation of HCO_3^- to CO_2 , the hydrogen ion concentration of the tubular liquid has in most cases been far below that of the plasma. Hence hydrogen ions must have been added to the liquid in the tubules in spite of the alkalosis.

It seems as if the "initial" pH values are the smaller the larger the diuresis. However, this may be merely due to the circumstance that a larger quantity of CO_2 has diffused back to the blood when the diuresis is small on account of the slower passage of the urine through the kidneys. Another factor may also play a rôle. If the urine is acidified at some place in the kidneys, the hydrogen ion concentration will here decrease until the transformation of HCO_3^- to CO_2 is equivalent to the added quantity of hydrogen ions. Since this transformation is proportional to the concentration of HCO_3^- it will proceed more rapidly at a small diuresis, and the equilibrium is then reached at a higher value of pH.

I have tried in vain to demonstrate in a direct way that the cells of the tubules supply hydrogen ions to the tubular liquid during alkalosis. The larger the buffer capacity of the urine the smaller will be the change in pH during the transformation of HCO_3^- to CO_2 . If we therefore greatly increase the buffer capacity there will be a possibility of obtaining acid urine during a weak alkalosis. I have tried to increase the buffer capacity in dogs by phosphate injections but while the animals could stand this treatment well during acidosis, during alkalosis the injection of phosphate led to strong clonic convulsions. The condition could be relieved by ingestion of calcium, but clearance decreased very considerably. I found that the hydrogen ion concentration decreased to about 6.2, but clearance was then at ca. 10 % of the

normal value. The experiments therefore do not constitute any reliable evidence.

In this connection it is worth mentioning that also under other conditions an acid urine has been found during alkalosis, and with low values of clearance. Thus NICOLS (1940) observed a decrease in the hydrogen ion concentration of the urine to about 6.0 during the final stage of alkalosis, where kidney injury occurs. This can be easily explained on the basis of the theory that the high values of pH under normal conditions are due to the transformation of HCO_3^- to CO_2 . When clearance is small only a small amount of bicarbonate is present in the liquid in the tubuli, and the transformation of HCO_3^- is therefore also small. Granted the correctness of this explanation we may expect to find acid urine also under other conditions, where the excretion of bicarbonate is reduced. This is in very good agreement with investigations which show a low value of the hydrogen ion concentration in the urine during alkalosis when there is at the same time a deficiency in sodium. Under these conditions most of the sodium is absorbed from the urine and therefore at the same time most of the bicarbonate.

On the whole the facts discussed above give some support to the idea that certain cells in the tubuli, without regard to the reaction of the organism, tend to make the urine acid. In the frog this has been proved to be the case in a direct way. ELLINGER (1940) has photographed the nephrons in frogs after ingestion of fluorescein and large amounts of bicarbonate, and found that in the liquid in the tubuli the hydrogen ion concentration was between 7.0 and 7.5 up to the last third of tubulus dist., where the concentration was between 6.0 and 6.5.

2. *The relation between p_{CO_2} and the diuresis.*

As shown in Fig. 1 the carbon dioxide tension during alkalosis decreases with increasing diuresis, provided the latter quantity exceeds 250 ml per hour. This may be explained by assuming the variations in the small diureses to arise by the excretion of more or less bicarbonate, the concentration in the urine being kept constant and close to its maximum value. The three values, with the diuresis smaller than 250 ml per hour and p_{CO_2} at the same time smaller than 105 mm, have been samples with a low concentration of bicarbonate.

It is therefore possible that if we were able to keep the excretion of bicarbonate per hour constant while varying the diuresis, we would find a relation between p_{CO_2} and the diuresis also when the latter quantity is less than 250 ml per hour.

The fact that the carbon dioxide tension decreases with increasing diuresis is after all not so surprising since the concentration of bicarbonate behaves in a similar way.

If the problem is considered on the basis of the aforementioned theory of the transformation of bicarbonate to carbon dioxide, it is seen that a smaller concentration of bicarbonate must entail a smaller transformation to CO_2 and hence a lower tension. Nothing is known about the changes in the process that tends to acidify the urine; if it proceeds unchanged it will of course bring about a transformation of HCO_3^- to CO_2 of the same order of magnitude as before *per hour*, but a smaller transformation *per liter*, and this will result in lower tensions. On the other hand there is no doubt that at a higher diuresis less carbon dioxide will diffuse back to the blood before the urine is emptied into the bladder, and this will tend to increase the tension. The situation is therefore very complicated and we must restrict ourselves to a statement of the final result: the carbon dioxide tension in urine during alkalosis decreases with increasing diuresis.

Finally it may be mentioned that still another explanation for the high carbon dioxide tensions is possible. Different nephrons may function differently, in such a way that some of them produce an acid urine, even during alkalosis. Mixing of this urine with the alkaline urine of the other nephrons brings about the increase in p_{CO_2} . The fact that there does not seem to be nephrons producing an alkaline urine during acidosis is probably not a decisive argument against this theory. The good reproducibility of the experimental results may well be due to a statistical regularity in the function of the nephrons entailing a considerable constancy in pH and p_{CO_2} from one experiment to another. However, it must be realized that if a scattering in the function of the nephrons should suffice to explain the high carbon dioxide tensions, then this scattering must be rather considerable.

Summary.

The carbon dioxide tension of the urine has been determined under different conditions; the hydrogen ion concentration has thereby been kept constant during each experiment.

In 30 experiments at $\text{pH} < 5.5$ the values of p_{CO_2} in the urine were 3.3 ± 2 mm higher than the values of p_{CO_2} in the alveolar air.

At $\text{pH} \approx \text{ca. } 6.5$ and with a large diuresis p_{CO_2} in the urine was ca. 15 mm higher than in the alveolar air.

At $\text{pH} > 7.0$ the difference between p_{CO_2} in urine and in alveolar air was still larger, from 20 to 70 mm, and it was found to decrease with increasing diuresis. When the diuresis was less than 250 ml per hour no relation could be found between diuresis and carbon dioxide tension in the urine.

It is pointed out that on account of the great diffusibility of CO_2 one would a priori expect the carbon dioxide tension in the urine to be almost equal to that in the blood. This is found not to be the case in alkaline urine, and to account for this two possible explanations are advanced: either, that the equilibrium between $[\text{HCO}_3^-]$ and $[\text{CO}_2]$ is established only after the urine has reached the collecting ducts, or, that the tubuli function differently, in such a way that some of them produce an acid urine even during alkalosis.

If the former theory is accepted, we are forced to the conclusion that the cells of the tubuli supply hydrogen ions to the urine even during alkalosis and that the urine becomes alkaline by the transformation of bicarbonate to carbon dioxide.

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Cardiovascular and Respiratory Changes in Man During Oxygen Breathing.

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When investigating the influence of oxygen upon respiration, arterial blood pressure and pulse frequency in experimental animals, v. EULER and LILJESTRAND (1942) observed a decrease in all three in the case of anaesthetized cats and dogs. The central rôle of the chemoreceptors in this respect is clear from the fact that a denervation of both sinuses extinguished the oxygen effect upon respiration. Neither was there any change in pulse frequency when sinus denervation was combined with vagotomy. After both sinus denervation and vagotomy all the animals exhibited a contrary reaction as regards the blood pressure, which rose. The same results have been obtained in experiments on rabbits (HEJNEMAN, 1943).

The effect upon respiration of breathing 100 % oxygen in the case of the human being has been described by several workers, but their observations seem rather contradictory. Thus in 6 cases DAVIES, BROW and BIGGER (1925) found an average increase in respiratory minute volume of 2.6 %. This deviation, however, was not statistically significant. BEHNKE, JOHNSON, POPPEN and MOTLEY (1935) observed no change in respiration frequency or respiratory minute volume induced by oxygen inhalation, in some cases during 4 hours. In these experiments, however, they did not begin the measurements until after the lapse of 80 minutes, and no information is given as to the immediate changes.

DAUTREBANDE and HALDANE (1921) stated that oxygen increases the ventilation, but they gave no further information. In the case of subjects breathing oxygen for 50 minutes, SHOCK and SOLEY (1940) found an increased respiratory minute volume. WATT, DUMKE and COMROE (1943) described an immediate increase in respiratory minute volume in 13 cases, a decrease in 4 and no change in 2. KEYS, STAPP and VIOLANTE (1943) maintained that the inhalation of oxygen at atmospheric pressure caused a slightly increased ventilation.

The influence of oxygen inhalation on the vital capacity has been studied by COMROE, DRIPPS, DUMKE and DEMING (1945). In 63 cases out of 80 they found a decline in vital capacity. The subjects breathed different oxygen concentrations (50, 75 and 100 %) in the course of 24 hours, and in most cases the decline was between 200 and 300 cc, but in 15 cases it reached 400 cc, with 2 peaks of 1,480 cc.

The results in respect of the pulse reaction during oxygen inhalation are more concordant. BENEDICT and HIGGINS (1911) demonstrated a pronounced decrease in pulse frequency, induced by the inhalation of high oxygen concentrations at atmospheric pressure. The same result has been described by DAUTREBANDE and HALDANE (1921), BEHNKE, SHAW, SCHILLING, THOMSON and MESSER (1934), ANTHONY (1940) and KEYS, STAPP and VIOLANTE (1943). By means of ballistocardiographic measurements WHITEHORN, EDELMANN and HITCHCOCK (1946) found a decrease in cardiac output, amounting to an average of 15.5 %. During the first 10 minutes this reduced cardiac output was almost exclusively due to a decreased pulse frequency and after that also to a decrease in stroke volume.

When reviewing the experiments performed by BEHNKE, SHAW, SCHILLING, THOMSON and MESSER (l. c.) in all cases except one BEAN (1945) found an increase in diastolic blood pressure, and in all cases except one a decrease in systolic blood pressure, thus a diminution of the pulse pressure. When studying the influence of oxygen breathing KEYS, STAPP and VIOLANTE (l. c.) demonstrated a slight, but statistically significant increase in the diastolic pressure and a tendency towards an increase in the systolic blood pressure. The pulse pressure decreased somewhat. WHITEHORN, EDELMANN and HITCHCOCK (l. c.) did not observe any change in the systolic blood pressure. On the other hand they found a slight, but definite increase in the diastolic pressure.

Method.

The subjects presented themselves early in the morning, without having taken any food during the preceding 12 hours. They were told to rest half an hour, either sitting (7 subjects) or lying down (8 subjects). A spirometer, containing 150 liters, was filled with air, the subject was connected up with the system and left so for another half hour, in order that he might get used to the apparatus and the respiratory conditions.

The experiment comprised 3 stages; 1. Breathing air for 20 minutes. 2. Breathing 100 % oxygen for 30—40 minutes. 3. Breathing air for 10 minutes. For the initiation of the oxygen breathing the subject was connected up with another spirometer, which had already been filled with oxygen, but he was then returned to the first spirometer, since this had been speedily filled with oxygen. This was done in order to maintain similar conditions during air and oxygen breathing.

In all 15 cases pulse and blood pressures were measured at intervals as shown in Table 3.

In 5 cases the respiratory frequency was measured and the expired air was collected in a Douglas bag, during periods of 10 minutes. In only 2 cases were the corresponding volumes measured during the third stage of the experiment.

In 12 subjects the vital capacity was measured, firstly during air breathing and secondly during oxygen inhalation. The measurements were performed with a Krogh spirometer, after the subjects had breathed into the above-mentioned spirometer. 18 measurements were made for each subject. The subjects were breathing oxygen for 30—40 minutes before the later measurements were made.

Results.

Expiration volume. In all cases we observed an increase in expiration volume during oxygen breathing. The increase in expiration volume in the second sample, which was taken after 20 minutes of oxygen breathing, was generally greater than the corresponding volume in the first sample, which was taken after 10 minutes of oxygen inhalation. On an average we found a 15.4 % increase in expiration volume. In the 2 cases in which the expiration air was measured during the third stage of the investigation, we found a pronounced decrease in both cases. Respiration frequency showed a certain, though insignificant, increase during oxygen inhalation. See Table 1.

Vital capacity. In 10 cases out of 12 we found a reduction of vital capacity during oxygen breathing. In the 2 remaining

Table 1.

Respiration Frequency and Expiration Volume.

Subject	Sitting = S Lying down = L	11 S	12 L	13 L	14 L	15 L				
After breathing	Respiration frequency/min. and Expiration volume in Liter/10 min.									
Air 10 min.	11	31.5	13	66.8	12	53.2	10	55.7	12	49.7
20 »	11	31.4	12	68.0	13	50.6	12	57.3	12	51.3
Average		31.5		67.4		51.9		56.5		50.5
100 % O ₂ 20 min.	12	33.9	13	69.0	13	62.7	13	64.6	13	56.5
30 »	13	36.5	14	77.2	14	66.5	14	69.2	13	58.6
Average		35.2		73.1		64.6		66.9		57.5
Diff. in %		11.7		8.5		24.5		18.4		13.9
Air 10 min.	—	—	—	—	—	—	12	63.9	12	54.8

Expiration volume: Average in % = + 15.4 \pm 2.793 $t = 5.514$ $P < 0.001$.

Table 2.

Vital Capacity.

Subject	Air	100 % O ₂	Diff. in cc.	Diff. in %
1	4.45	4.20	— 250	— 5.7
2	5.80	5.40	— 400	— 6.9
3	4.75	4.50	— 250	— 5.3
4	4.10	3.90	— 200	— 4.9
5	5.20	4.90	— 300	— 5.8
6	3.95	4.25	+ 300	+ 7.5
7	5.20	5.15	— 50	— 1.0
8	4.52	4.32	— 200	— 4.5
9	4.70	4.60	— 100	— 2.2
10	4.65	4.40	— 250	— 5.4
11	5.35	5.50	+ 150	+ 2.8
12	5.05	4.80	— 250	— 5.0

Average in % = — 3.03 \pm 1.229 $t = 2.465$ $0.02 > P > 0.05$.

cases we found an increase. The reduction amounted at most to 6.9 % and on an average to 3.03 %, with a statistical significance between 0.98 and 0.95. The greatest reduction amounted to 400 cc. See table 2.

Pulse frequency. In all cases we found a pronounced reduction of pulse frequency, averaging 9.1 %. The effect of oxygen breath-

ing was striking, and even after 2 minutes the lowest value had been reached in 5 cases, and in most of the other cases this occurred after 5 minutes. The pulse frequency, too, was taken in the third stage and showed an immediate increase in all cases. See table 3.

Blood pressure. The diastolic blood pressure showed an increase in 12 cases, in 2 cases amounting to somewhat more than 10 %. In 2 cases we found a slight decrease, and in 1 case the diastolic pressure remained unchanged. Statistical calculations showed a slight but significant increase in diastolic blood pressure, amounting to 5.7 %. In 10 cases the systolic pressure was slightly increased, in 3 cases slightly reduced and in 2 cases unchanged. There was a slight tendency towards an increase. The pulse pressure showed a reduction in 10 cases, an increase in 4, and was unchanged in 1 case. The mean pressure was slightly increased. See table 3.

Discussion.

The anaesthetization of experimental animals causes a certain hypoxaemia, resulting in increased respiration, pulse frequency and blood pressure. The supply of oxygen removes the oxygen deficiency as a stimulous and thereby causes a decrease in all these phenomena.

The contrary effect on respiration and blood pressure caused by the oxygen, when acting at a peripheral and central point, is obvious from sinus denervation in animals (v. EULER and LILJESTRAND, 1942). If the chemoreceptors are intact, oxygen consistently causes a decrease in ventilation, while after denervation there is sometimes an increase. This is assumed to be due to the direct influence of the oxygen on the respiratory centre, the anoxaemia, resulting on the decreased breathing after sinus denervation, being reduced.

The changed reaction of the blood pressure before and after sinus denervation is explained in the same way. The blood pressure is reduced after denervation during hypoxaemia, as has been shown by v. EULER and LILJESTRAND (1936), and these authors (1942) are of the opinion that a certain degree of hypoxaemia appears in the breathing in ordinary air after sinus denervation. In this case the inhalation of oxygen will only act directly upon the vasomotor centre and result in an increase in tone.

The lessened pulse frequency during oxygen breathing, when the chemoreceptors are intact, is explained as being due to a moderation of the impulses from the sinus receptors to the central regulating organs, the impulses being more frequent during hypoxaemia. A certain effect also remained, however, after total denervation of the sinus (v. EULER and LILJESTRAND, 1942).

The reducing effect of the oxygen upon respiration, with the chemoreceptors intact, is however only of short duration. The increase in respiration after this is explained (v. EULER and LILJESTRAND, 1942) as being due to a higher carbon dioxide tension in the blood, caused by the reduced ventilation at the commencement of the oxygen breathing.

In experiments on unanaesthetized human beings the first phase of relative oxygen deficiency is absent. As a result of the oxygen inhalation, there is a great increase in the physically dissolved oxygen. Thus transported, the oxygen will in part supply the oxygen requirements of the tissues. There will be a decrease in the available reduced haemoglobin and as a result a reduction in the supply of cations for the transport of carbon dioxide. In this case a certain addition of acid metabolites would take place in the tissues. The increase in the oxyhaemoglobin would also cause greater acidity in the blood. According to SHOCK and SOLEY (l. c.) the impaired transport of carbon dioxide explains the increased ventilation during oxygen inhalation, which would thus be ascribed to a greater carbon dioxide stimulation of the breathing centre.

COMROE, DRIPPS, DUMKE and DEMING (l. c.) discuss very fully the cause of the decrease in vital capacity during oxygen breathing. That the cause is a substernal pain, sometimes observed in the case of oxygen breathing, is rejected on the grounds that most subjects do not feel any such pain. Atelectasis, pulmonary engorgement and incipient pulmonary oedema were considered, but x-rays showed there could be no such possibility. These authors consider the cause to be alveolar damage. No sign of any such damage in form of a decrease in arterial oxygen tension was, however, to be found.

Nor can that explanation be acceptable in our cases, and it appears hardly probable that 30—40 minutes' oxygen breathing would cause such damage to the alveolar epithelium as to lead to the reduction in vital capacity met with in our cases. We should prefer to consider the possibility of an accumulation of

Table 3.
Pulse Frequency and Blood Pressure.

Subject Sitting = S Lying down = L	1 S	2 S	3 S	4 S	5 S	6 S	7 L							
After breathing	Pulse/min. and Blood pressure in mm. mercury													
Air -- min. . . .	67.4	116/68	59.4	106/68	60.0	112/70	61.2	110/82	64.5	102/88	60.0	110/78	67.4	120/86
10 » ..	65.2	126/78	61.2	106/68	60.0	110/72	60.0	110/84	65.9	102/88	58.8	112/78	72.3	118/84
20 » ...	63.8	116/80	58.8	107/70	58.8	112/78	57.7	110/84	63.1	102/88	57.7	113/80	66.7	118/84
Average	65.5	119.3/75.3	59.8	106.3/68.7	59.6	111.3/73.3	59.6	110.0/83.3	64.5	102.0/88.0	58.8	111.7/78.7	68.8	118.7/84.3
100 % O ₂ 2 min.	55.5	112/78	53.5	112/74	51.3	110/78	51.7	114/84	60.0	102/86	53.5	120/84	65.2	118/86
5 »	56.6	120/84	54.5	112/80	50.8	113/80	53.1	116/86	60.0	102/86	55.5	112/80	63.8	122/88
10 »	53.5	122/84	55.0	114/74	54.5	116/82	51.7	116/86	58.8	102/88	54.5	112/80	63.8	122/90
20 »	54.5	122/86	53.1	114/74	56.0	117/84	52.1	114/84	57.1	102/92	54.5	114/84	64.0	120/86
30 »	52.1	124/86	52.6	108/70	58.8	116/86	56.6	112/80	57.7	102/78	55.0	113/82	65.2	122/86
40 »	52.6	116/82	52.1	110/80	58.8	122/78	50.8	110/82	59.4	102/92	54.2	114/82	63.1	120/88
Average	54.1	119.3/83.3	53.5	111.7/75.3	55.0	115.7/81.3	52.7	113.7/83.7	58.7	102.0/88.5	54.5	114.2/82.0	64.2	120.7/87.3
Diff. in %	-17.4	0.0/10.6	-10.5	5.1/9.6	-7.7	3.9/10.9	-11.6	3.4/0.5	-9.0	0.0/0.6	-7.3	2.2/4.2	-6.7	1.7/3.6
Air 2 min.	59.4	118/82	57.1	110/68	59.4	122/84	52.6	110/82	61.2	104/64	58.2	120/84	65.2	122/84
5 » ...	56.6	124/88	58.2	112/70	63.1	122/84	56.6	110/82	61.8	104/68	60.0	114/86	64.5	122/86
10 » ...	56.6	123/86	58.2	110/70	63.1	116/78	57.7	110/82	63.1	105/68	60.0	110/80	65.2	122/88

Subject Sitting = S Lying down = L	8 L	9 L	10 L	11 S	12 L	13 L	14 L	15 L								
Pulse/min. and Blood pressure in mm. mercury																
After breathing																
Air — min.	59.4	106/64	81.1	136/82	67.4	120/80	75.0	118/72	61.2	124/86	50.8	127/80	69.7	128/84	68.9	132/74
10 » ...	58.8	112/70	80.0	138/82	67.4	120/78	74.0	118/70	60.0	120/78	50.8	130/84	73.2	129/86	75.0	130/74
20 » ...	60.0	114/78	77.9	136/84	65.2	122/78	73.2	118/76	59.4	118/78	50.8	132/80	73.2	126/88	68.9	132/74
Average	59.4	110.7/70.7	79.7	136.7/82.7	66.7	120.7/78.7	74.1	118.0/72.7	60.2	120.7/78.6	50.8	129.7/81.3	72.0	127.7/86.0	70.9	131.3/74.7
100 % O ₂ 2 min.	52.6	120/76	75.0	142/86	61.8	120/80	65.9	116/72	56.0	114/80	45.8	136/88	67.4	129/86	66.7	134/77
5 »	50.8	116/76	74.0	142/86	60.0	122/78	66.7	114/72	55.5	124/76	48.8	132/84	65.2	130/86	68.9	132/74
10 »	54.0	114/74	75.0	140/90	60.0	120/80	63.0	115/72	51.7	116/78	49.1	135/90	67.4	126/88	66.7	132/72
20 »	54.0	116/74	74.2	136/76	58.8	124/80	61.2	116/72	55.0	124/76	49.6	134/90	69.0	126/86	68.9	134/74
30 »	54.5	118/78	72.3	132/84	58.8	124/80	62.5	114/74	53.1	122/72	47.6	126/86	70.6	126/86	68.2	136/74
40 »	55.5	118/74	73.2	136/78	58.8	120/80	—	—	—	—	—	—	—	—	—	—
Average	53.6	117.0/75.3	73.9	138.0/83.7	59.7	121.7/79.7	63.9	115.0/72.4	54.3	120.0/79.3	48.2	132.6/87.6	67.9	127.4/86.0	67.9	133.6/73.6
Diff. in %	-9.8	5.7/6.5	-7.3	0.9/1.2	-10.5	0.8/1.3	-13.5	-2.5/-0.4	-9.8	-0.6/0.9	-5.1	2.2/1.7	-5.7	-0.4/0.0	-4.3	1.7/-1.5
Air 2 min.	58.2	118/76	75.0	134/78	61.8	122/80	67.4	114/74	57.1	122/74	55.0	134/88	72.3	130/80	74.1	132/74
5 » ...	60.0	116/74	74.0	132/78	62.5	120/78	67.4	116/74	65.2	124/76	56.6	130/80	70.6	128/82	75.0	134/74
10 »	58.8	114/76	74.0	132/78	61.8	118/78	68.2	115/72	64.5	122/78	53.8	134/84	70.6	130/86	70.6	132/74

Pulse frequency: Average in %: -9.1 ± 0.892 $t = 10.20$ $P < 0.001$

Syst. blood pressure: Average in %: $+1.6 \pm 0.578$ $t = 2.76$ $0.01 > P > 0.02$.

Diast. blood pressure: Average in %: $+5.7 \pm 1.065$ $t = 5.35$ $P < 0.001$.

blood in the depots, which would also explain the decrease in vital capacity. In experimental animals v. EULER and LILJESTRAND (1946) demonstrated that breathing of pure oxygen lowered the pulmonary arterial pressure.

The reduction of the pulse pressure found in some of our cases, is in accord with the results obtained by BEHNKE, SHAW, SCHILLING, THOMSON and MESSER (l. c.), KEYS, STAPP and VIOLANTE (l. c.) and WHITEHORN, EDELMANN and HITCHCOCK (l. c.). A combination of reduced pulse pressure and reduced minute volume (WHITEHORN, EDELMANN and HITCHCOCK, l. c.) indicates a general peripheral vasoconstriction. A vasoconstriction has also been shown directly by a study of the blood vessels of the eye during oxygen breathing (ROSENTHAL, 1939). These findings do not seem to conform with SHOCK and SOLEY's theory of the impaired transport of the carbon dioxide, as in that case all the tissues would obtain an excess of carbon dioxide, which in its turn would cause general vasodilatation. Neither do these statements conform with the above-mentioned hypothesis of an accumulation of blood in the depôts. A central influence of the carbon dioxide on the vasomotor centre might however counteract or suppress such peripheral carbon dioxide action. The influence of varying carbon dioxide tensions has been investigated in animal experiments by v. EULER and LILJESTRAND (1946). In animals with intact chemoreceptors they at first obtained a slight fall in the blood pressure, followed by a slight rise. This effect was much more pronounced after sinus denervation. The reaction is interpreted as being due to a peripheral CO₂-action, followed by a central CO₂-stimulation.

Summary.

The effect upon respiration, pulse frequency and blood pressure in man during the inhalation of 100 % oxygen at atmospheric pressure has been studied.

Expiration volume. In all cases an increase, averaging 15.4 %.

Respiration frequency. In all cases an inconsiderable increase.

Vital capacity. In most cases a decrease, averaging 3.03 %.

Pulse frequency. In all cases an almost immediate decrease, averaging 9.1 %.

Blood pressure. In most cases a decrease in pulse pressure. The diastolic blood pressure showed a statistically significant increase

of, on an average, 5.7 %. The systolic pressure was almost constant.

No toxic symptoms were observed during these experiments.

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Interaction between Acetylcholine and Adenosine Triphosphate in Normal, Curarised and Denervated Muscle.

By

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In previous communications it has been shown, that adenosine triphosphate (ATP) when applied in small amounts to amphibian and mammalian muscle, apart from releasing contraction, has a sensitizing effect on subsequent application of acetylcholine. The sensitivity of a muscle to acetylcholine is increased four to ten times. Inorganic triphosphate has the same effect (BUCHTHAL et al. 1944, BUCHTHAL and FOLKOW 1944, BUCHTHAL and KAHLSON 1944). It was left undecided, whether the changes observed were connected with the neuromuscular transmission, *i. e.* were specific for a stimulation by acetylcholine, or whether they were a property of the contractile substance.

While in normal muscle previous application of acetylcholine has no effect on subsequent contractions released by ATP, the opposite was shown to be the case in denervated mammalian muscle, in which a preceding application of acetylcholine makes the muscle insensitive to intra-arterially applied ATP (BUCHTHAL and KAHLSON 1946). Also in denervated frog's muscle a previous application of acetylcholine abolishes the release of contraction by ATP (BUCHTHAL et al. 1946). No explanation could be offered for this peculiar effect in denervated muscle.

It is the aim of the present investigation to study the relations between ATP, acetylcholine and electrical stimulation in normal

and denervated muscle by using the height of contraction, the threshold and the strength-duration curves as measure of the changes induced by chemical stimulation.

Method.

Acetylcholine and ATP were applied by intra-arterial injection through a fine glass cannula, which was inserted in the sciatic artery of the frog, immediately above the branching of the artery in the popliteal fossa (BROWN 1937). The mechanical response of the gastrocnemius was recorded, after branches of the artery supplying other muscles had been ligated. In some experiments the substances were applied intramuscularly under careful avoidance of mechanical disturbances. It was regularly checked that even large amounts of Ringer solution did not produce any mechanical response. ATP prepared from Ba and Ca salts was applied as the Na-salt in an iso-osmotic solution with a pH of 7.3. All arterial injections were made in a volume of 0.03 ml Ringer. The Ringer solution contained per liter 6.3 g NaCl, 0.2 g KCl, 0.1 g anhydric CaCl_2 , 0.2 g glucose and 3 per cent dextran to obtain normal colloid-osmotic pressure. A pH of 7.3 and saturation with O_2 was obtained by a suitable amount of NaHCO_3 and by passing a gas mixture of 1 per cent CO_2 and 99 per cent O_2 through the solution. The mechanical responses were registered by means of a torsion band myograph and photographically recorded. Electrical stimulation, which was applied to the muscle by thin silver wires, consisted of rectangular current pulses of adjustable voltage and duration. The frequency of stimulation was 1—2/sec. Curarine was likewise applied intra-arterially and its effect tested by the disappearance of indirect excitability. For experiments on denervated muscles the sciatic nerve was cut on one side immediately after its passage through the greater sciatic foramen and degeneration was allowed to proceed for a period of three to four weeks. In another series of experiments acetylcholine and ATP were applied directly on isolated non-curarised and curarised muscle fibres before and after denervation. All experiments were performed at a temperature of 18—20° C.

Results.

1. *Acetylcholine and electrical excitability.* The effect of intra-arterially applied acetylcholine is different in summer- and winterfrogs. In the former the threshold dose is about 1/10th of that in winterfrogs. The mechanical response in summerfrogs is of short duration (twitch-like) while in winterfrogs the contraction generally is of the contracture-like type, even when the substance is applied in only threshold amounts. These findings

correspond to observations by WACHHOLDER and NOTHMANN (1932) and WACHHOLDER and LEDEBUR (1933) who found differences in the reaction of summer- and winterfrogs to large doses of acetylcholine, which were added to the surrounding Ringer bath. In spite of the different reaction to acetylcholine, the mechanical response to electrical stimuli is identical. When

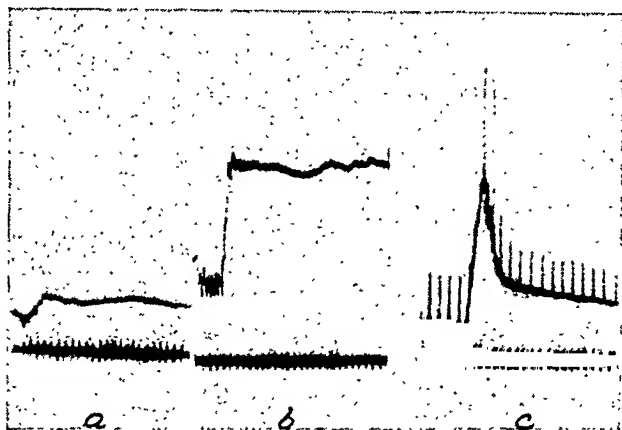


Fig. 1. a) Winterfrog, 1 μ g acetylcholine
 b) » 4 μ g »
 c) Summerfrog 0.5 μ g »

Acetylcholine applied by close arterial injection. During the contraction responses produced by electrical stimulation. Note the reduction of these superimposed contractions in b and their enhancement in c.

the muscle is periodically stimulated during the acetylcholine contraction the superimposed contractions are depressed, as also found by BROWN (1937). This is, however, only the case in winterfrogs. In summerfrogs the opposite is seen, the contractions produced by electrical stimulation of the muscle being considerably enhanced on the top of the acetylcholine response (Fig. 1).

Strength-duration curves: The effect of acetylcholine in the surrounding bath on the strength-duration curves of frog's sartorius muscle has previously been investigated by BLAIR (1938) and KUFFLER (1945). Subthreshold concentrations were found to be without effect while amounts which produced contractions (5 μ g/ml) resulted in an increase of chronaxie and a decrease of the rheobase. Intra-arterial application of acetylcholine gives quite different results. After injection of 0.5 to 1 μ g acetylcholine the threshold increases by about 50 per cent. The increase is the same for all impulse durations investigated. (Fig. 2, curves I

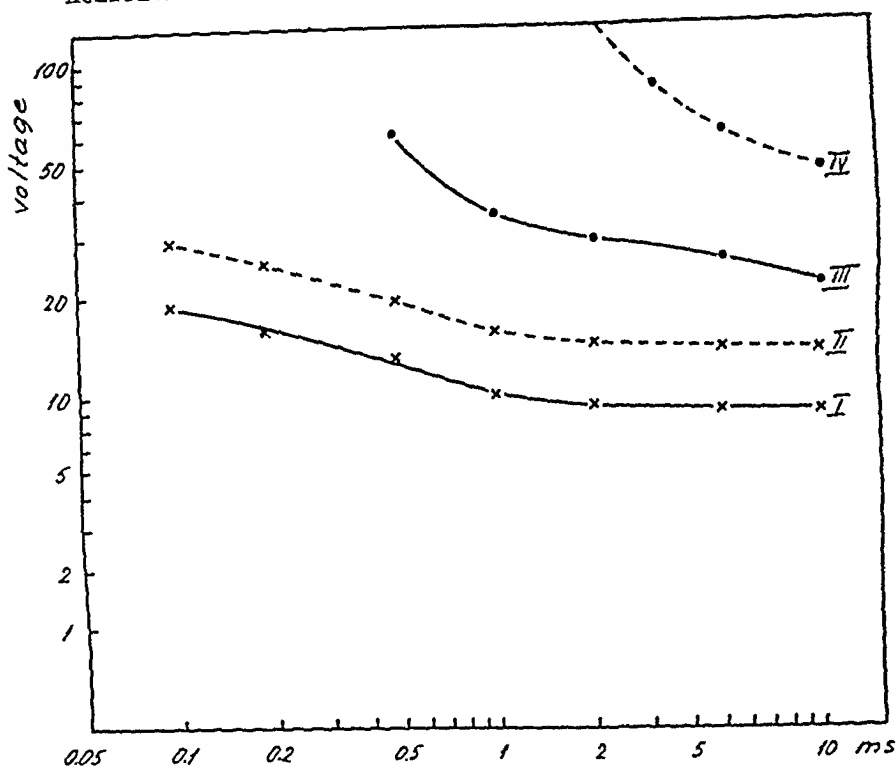


Fig. 2. Strength-duration curves before and after close arterial injection of acetylcholine in normal and denervated muscles of the same animal.

I) Normal "muscle" (intramuscular nerves) before application of acetylcholine.
 II) Normal "muscle" (intramuscular nerves) after application of $1.5 \mu\text{g}$ acetylcholine.

III) Denervated muscle before application of acetylcholine.

IV) Denervated muscle after application of $0.15 \mu\text{g}$ acetylcholine.

Ordinate: Voltage in relative units.

Abscissa: Time in ms. Both in logarithmic scale.

and II.) When acetylcholine is injected into a denervated muscle, the decrease in excitability is considerably greater. (Fig. 2, curves III and IV.) With a stimulus duration of 10 ms it amounts to 100 per cent, increasing to 300 per cent above the value for the untreated denervated muscle with a stimulus duration of 2 ms, and the threshold is 15 times above that of normal muscle. The amounts of acetylcholine necessary to produce this effect are only 1/10th of those which are needed in normal muscle. Similar changes in excitability and in the strength-duration curves occur in curarised muscle after large amounts of acetylcholine (10—15 μg). The curare-like effect of acetylcholine on repeated application is also more pronounced in the denervated than in the normal muscle.

2. *Adenosine triphosphate and its interaction with acetylcholine.* The amounts of intra-arterially applied ATP which are needed to elicit a contraction are independent of whether the muscle is curarised or denervated. In summerfrogs 1 μg acetylcholine corresponds approximately to 40–50 μg ATP, in winterfrogs sometimes larger amounts of ATP are necessary. In normal

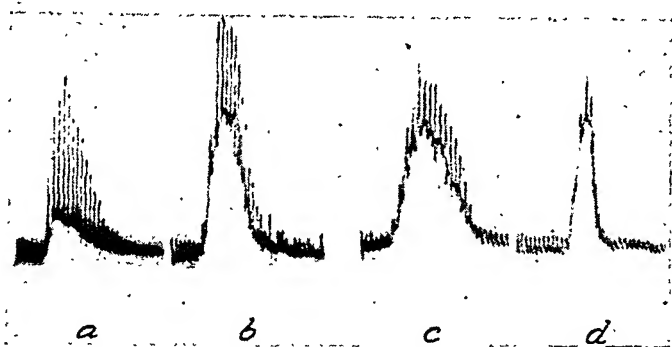


Fig. 3. Close arterial injection of ATP in normal, curarised, denervated and curarised denervated muscle of the same animal.

Note the enhancement of responses released by electrical stimulation during the ATP-contraction.

a) Denervated muscle, 100 μg ATP.

b) Curarised denervated muscle, 250 μg ATP.

c) Normal muscle, 200 μg ATP.

d) Curarised muscle, 250 μg ATP.

Frequency of electrical stimulation 2/sec.

muscle small twitches are superimposed on the response evoked by ATP. These correspond to the repetitive asynchronous activity which was observed after application of ATP to the isolated muscle fibre. Curarisation reduces the superimposed twitches and at the same time the duration of the ATP contraction is considerably diminished. In normal, curarised, denervated and curarised denervated muscle the electrically induced mechanical responses, which are superimposed on the ATP contraction, are enhanced in the ascending phase and on the top of contraction, while they are slightly depressed during relaxation (Fig. 3).

Thus, the sensitizing effect of ATP is not specifically connected to the neuromuscular transmission, but is a property of the muscular substance. It is, however, worth mentioning that the increased excitability to electrical stimulation is restricted to the actual period of contraction and that it is followed by a slight depression, while the sensitizing effect for acetylcholine may persist for 5–15 minutes. Apart from its sensitizing action on subsequent

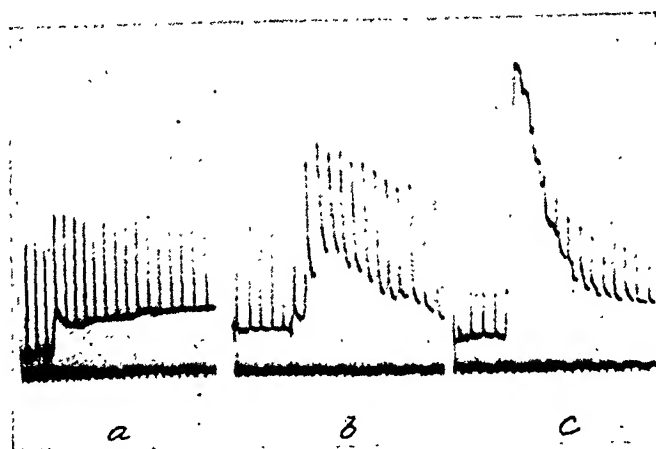


Fig. 4. Winterfrog. The contractions produced by close arterial injections of acetylcholine and ATP are superimposed by mechanical responses released through electrical stimulation.

a) 6 μ g acetylcholine.

b) 250 μ g ATP.

c) 5 μ g acetylcholine.

Note the change of the acetylcholine response after treatment with ATP, the contraction, apart from being much larger, is of a more twitch-like type after ATP.

Distance between time marks 1 sec.

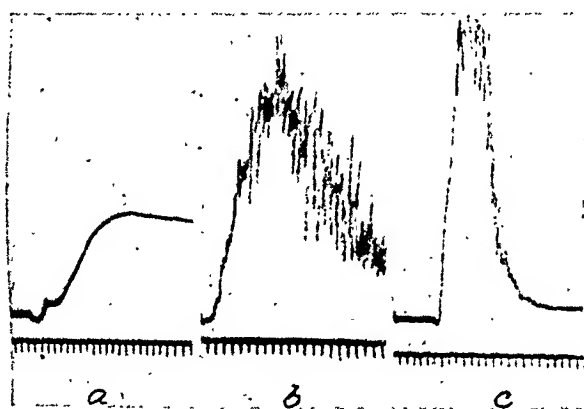


Fig. 5. Denervated muscle. Contractions released by close arterial injection of acetylcholine and ATP.

a) 0.3 μ g acetylcholine.

b) 300 μ g ATP (strong fibrillation).

c) 0.3 μ g acetylcholine.

Note the change of the acetylcholine response after treatment with ATP; the contraction becomes much larger and is of a shorter duration.

Distance between time marks 1 sec.

application of acetylcholine or electrical stimulation, ATP transforms the contracture-like contraction of long duration found in winterfrogs and in denervated muscles into a short tetanic response (Fig. 4 and 5). Even in curarised muscle the acetylcholine con-

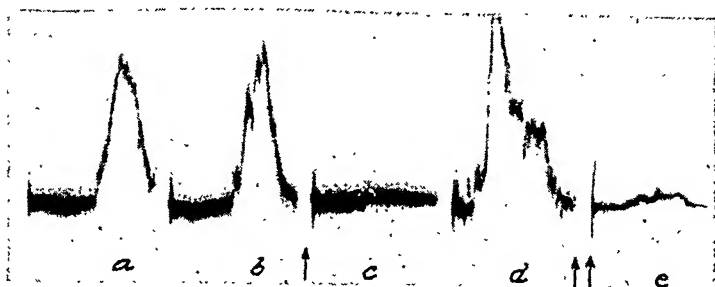


Fig. 6. Denervated muscle.

- a) intra-arterial ATP 300 μ g.
- b) as a.
- ↑ intra-arterial acetylcholine 0.1 μ g.
- c) intra-arterial ATP 600 μ g.
- d) intramuscular ATP, 30 μ g. (Control with the same amount of Ringer had no effect.)
- ↑↑ intra-arterial acetylcholine 3 μ g.
- e) intramuscular ATP. 600 μ g.

tracture may be transformed into a contraction of short duration with increased amplitude. The strength-duration curves of normal and denervated muscle are unaffected or slightly depressed after treatment of the muscle with ATP.

The blocking action of acetylcholine to subsequent application of ATP observed in denervated mammalian muscle is also found by close arterial injection into frog's muscle. Injection of 0.1 μ g acetylcholine inhibits the release of contraction by even large amounts of ATP. In order to decide whether this block is localised to the interaction between myosin and ATP or is due to changes caused by acetylcholine elsewhere on the way from the artery to the muscle fibre thereby preventing contact between ATP and muscle, we have injected ATP directly into a muscle which is insensitive to intra-arterially applied ATP (Fig. 6). This results in a strong response in the denervated muscle. Also in curarised normal muscle acetylcholine, inactive as such blocks the action of intra-arterially applied ATP, though the responsiveness to electrical stimuli is retained. When ATP is injected directly into the muscle a strong contraction is released. Injection of Ringer solution in the same amount is without effect. In curarised denervated muscle we find similar conditions.

When the amount of acetylcholine is increased 10–30 times (1–3 μ g) also intramuscular injection of ATP is without effect, thus indicating that the blocking action of large amounts of acetylcholine is localised in the muscular substance (Fig. 6).

This observation corresponds with the results obtained on isolated fibres from denervated muscles in which we find a considerable rise in threshold for ATP¹ after previous application of 1—25 μ g acetylcholine while smaller amounts of acetylcholine are without effect.

Discussion.

The sensitizing action of ATP on subsequent application of acetylcholine is shown to be a property of the muscular substance, since also the response to electrical stimuli even in curarised muscle is enhanced by ATP. There is, however, a considerable difference in the duration of the sensitizing effect, the threshold for acetylcholine being lowered for several minutes after the application of ATP, while the electrically induced contraction is increased only during the period of contraction. In terms of minute structure this sensitizing effect would mean an increased readiness of contractile elements in the fibres to receive stimulation quanta, resulting in an increased number of contracted elements per stimulus. It corresponds to the differences in tension produced by a single stimulus and by a tetanic stimulation, as the tension developed is determined by the number of stimulation quanta released in a given time (BUCHTHAL and KAISER 1944).

The blocking action of acetylcholine on subsequent intra-arterial application of ATP in denervated and curarised muscle occurs in two stages according to the amounts of acetylcholine injected. The reason why small amounts of acetylcholine prevent ATP administered through the artery to produce a response in denervated and curarised muscle remains obscure (vascular permeability?). Further investigations will be necessary to decide whether this effect is due to the partial autonomic denervation. In larger amounts acetylcholine also inhibits the release of contraction by intramuscularly applied ATP. As the electrical excitability is retained, acetylcholine in some way or other must be assumed to prevent a contact between ATP and the contractile elements in the muscle.

¹ Our statement in a previous paper (BUCHTHAL et al. 1946) that acetylcholine abolished the effect of ATP in denervated amphibian muscle has to be modified, since the present experiments show that there is no total block, but a considerable rise in threshold for ATP.

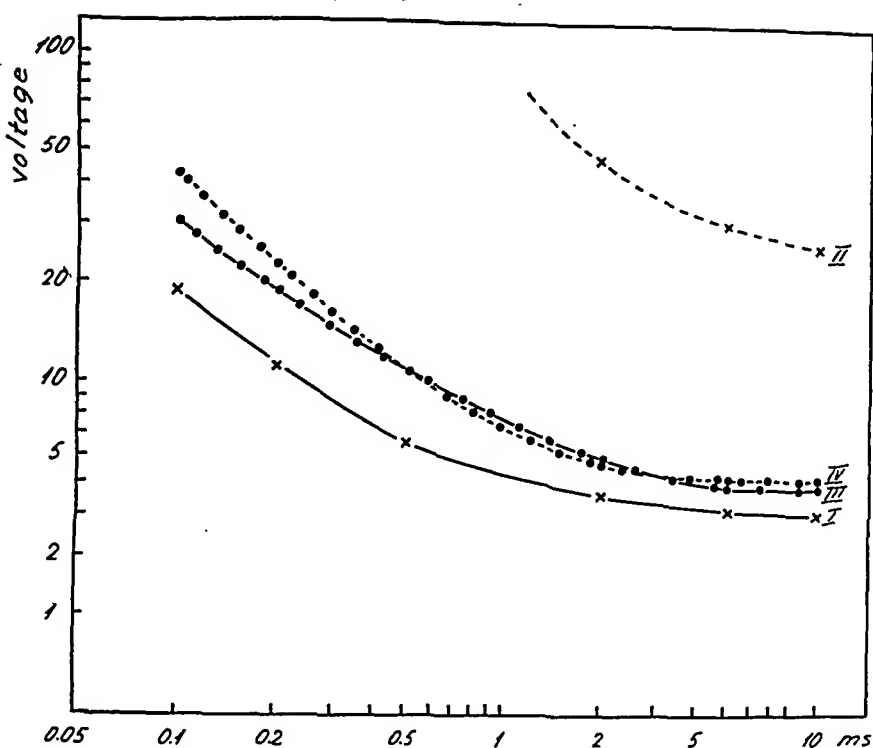


Fig. 7. Strength-duration curve before and after curarisation in normal and denervated muscle of the same animal.

I) Normal "muscle" (with intramuscular nerves).

II) Normal "muscle" after curarisation.

III) Denervated "muscle" before curarisation.

IV) Denervated "muscle" after curarisation.

Ordinate: Voltage in relative units, abscissa: Time in ms. Both in logarithmic scale.

The same causes which are responsible for the missing effect of ATP after previous application of acetylcholine can possibly explain a number of differences which have been found between the effects of chemical and electrical stimulation. It is necessary to bear in mind that both close arterial injection and direct application of acetylcholine to the endplate imply the passage through other interfaces than those involved in the physiological liberation of a possible chemical transmitter by electrical stimulation.

A comparison between strength-duration curves of curarised normal and curarised denervated muscle shows a considerable difference, the electrical excitability being much greater in the latter than in the former. Denervated and curarised denervated muscle have almost identical curves (Fig. 7). This is in agreement

with the results of KUFFLER (1945) who fails to find any specific electrical excitability of the endplate region in frog muscle. On the other hand the difference in electrical excitability between denervated and curarised normal muscle and the increased excitability of the endplate in lizard muscle (BUCHTHAL and LINDHARD 1939) are hardly consistent with this interpretation.

Summary.

1. Application of adenosine triphosphate (ATP) preceding the intra-arterial injection of acetylcholine increases the sensitivity of striated amphibian and mammalian muscle to acetylcholine. This sensitization is not specifically connected with the neuromuscular transmission, for there is also in curarised muscle an increase of the mechanical response produced by electrical stimulation after previous application of ATP. This increase is, however, restricted to the actual period of contraction.

2. In *denervated* muscle intra-arterial injection of acetylcholine renders the muscle insensitive to subsequent application of ATP. When small amounts of acetylcholine are applied, the block is only present when ATP is administered through the artery, intramuscular application of this substance still being highly effective in the release of contraction.

Larger amounts of acetylcholine also prevent intramuscularly applied ATP from being active. The electrical excitability remains unaltered.

3. The threshold for intra-arterially injected acetylcholine was found to be ten times higher in winterfrogs than in summerfrogs. In the former acetylcholine produces a contracture-like response. Previous application of ATP changes it to a contraction of short duration. When twitches are produced by electrical stimulation during the acetylcholine response, they are enhanced in summerfrogs and reduced in height in winterfrogs.

4. Intra-arterial injection of acetylcholine decreases the electrical excitability and changes the strength-duration curve of muscle for a considerable time after its application. The effect is especially pronounced in denervated muscle. The electrical excitability of denervated muscle is considerably higher than that of curarised muscle, while strength-duration curves of denervated muscle are unaffected by curarisation. Previous application of ATP does not affect the strength-duration curve of a muscle.

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The Importance of Sodium Ions for the Excretion of Ammonium and Hydrogen Ions in the Urine.

By

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Among many other important functions the kidneys also act as regulators of the acid-base equilibrium of the body by eliminating from the organism a varying quantity of H^+ ions. This comes about partly through the binding of more or less H^+ ions to the buffer systems in the urine, and partly through the production in the kidneys of varying amounts of NH_3 , which binds H^+ ions under the formation of NH_4^+ .

By experiments with perfusion of single tubules in frog kidneys MONTGOMERY and PIERCE (1936) have shown that the perfusion liquid changes its pH from 7.5 to less than 6.7 when brought into contact with a distal part of the second order tubules.

WALKER (1940) has found that the formation of ammonia takes place in the same part of the tubule. It is therefore a rather obvious idea that, besides the formation of ammonia, another process also occurs here, with the purpose of supplying H^+ ions to the ammonia as well as to the buffer systems of the urine.

It must be remarked here that there are no reasons for assuming the existence of special processes in the tubular cells which make the urine alkaline during alkalosis. As mentioned in a previous paper (RYBERG 1948 b) there are indications that the cells of the tubules even during alkalosis produce an acid urine, which becomes alkaline only by transformation of HCO_3^- to CO_2 after the urine has left the tubules. There is therefore nothing to prevent that the

variations in the hydrogen ion concentration of the urine may well be regulated by a single process, which gives off H^+ ions to the tubular liquid.

PITTS and ALEXANDER (1945) have injected large amounts of phosphate into dogs during acidosis and shown "that the quantity of acid excreted in the urine may far exceed the quantity of acid filtered through the glomeruli. Therefore the urine must be acidified by some renal tubular mechanism which adds H^+ ions to the glomerular filtrate." They consider it most likely that an exchange takes place between Na^+ ions in the tubular liquid and H^+ ions in the cells of the tubules.

If the assumption is correct that hydrogen ions are exchanged with cations from the urine it is natural to think that the ammonium ions are transferred to the urine in a similar way. If this is the case it must be expected that a larger formation of ammonia gives rise to a larger reabsorption of positive ions; it is further to be expected that the excretion of ammonium ions will depend upon whether positive ions are at disposal for the exchange, and at most this excretion can be equivalent to the total amount of cations reaching the cells of the distal tubule per unit time.

The main purpose of the present work has been to investigate whether it is possible by decreasing the excretion of cations in the urine during acidosis to bring about a decrease in the formation of ammonia and hydrogen ions. It was also considered important to investigate whether any particular cation plays a dominant rôle in this connection.

While the excretion of ammonia can be easily determined, it is not possible quantitatively to measure the amount of hydrogen ion delivered to the tubular fluid. The titratable acid of the urine will not equal this amount, since some of the hydrogen ions will have been spent in converting HCO_3^- to CO_2 (cf. PITTS and LOTSPREICH (1946) and RYBERG (1948 b)). In the experiments pH in the urine has been taken as a rough indication of the intensity with which the acidifying processes work.

Methods.

Determination of ammonia in urine. The determinations were carried out by means of permutite according to a method already described in a previous paper (RYBERG 1948 a). This method has the advantage that the results are in no way influenced by the presence of the other

cations in the urine, a feature of particular importance in the present work.

Determination of sodium and potassium in urine. It turned out to be necessary to carry out an ashing process on the urine, since in many cases we had to determine very small concentrations of sodium and correspondingly large samples of urine had to be used.

0.4 ml conc. sulphuric acid were added to a suitable quantity of urine; in samples with low content of sodium up to 20 ml urine were used. The samples were carefully evaporated in quartz crucibles without cover on a heating plate. The ashing was then carried out, with a lid placed over the crucibles, in an electric oven, 1 hour at 250°, 1 hour at 350° and finally 4 hours at 600°.

The ashes were dissolved by heating with 5 ml distilled water, and the phosphate was precipitated by addition of 0.2 g. CaO. The precipitate was removed by filtering into a 25 ml measuring flask and rinsing carefully five times with 3 ml water. The measuring flask was now filled up to the mark and the solution used for the determination of both sodium and potassium.

For the determination of sodium 10–20 ml of the solution were evaporated in a Hagedorn tube, and the residue was dissolved in 1 ml water. Then at least 10 ml zinc uranyl acetate, prepared according to BARBER and KOLTHOFF (1940), were added and the determination carried out as described by these authors. A blank was always carried out.

We have made no use of the alcoholic precipitation reagents. These reagents certainly reduce the solubility of sodium zinc uranyl acetate, but simultaneously the solubility of potassium is so strongly reduced that we get, at any rate when 50 per cent alcohol is used, an almost quantitative precipitation of potassium.

With the precipitation reagent used by us 15 mg potassium (as potassium sulphate) could remain in the solution per 10 ml precipitation reagent. Since the concentration of potassium in all the urines with a low content of sodium has been below 2 mg per ml, we have, in order to avoid precipitation of potassium, always used 10 ml precipitation reagent corresponding to the salt content from 7 ml urine.

We have determined the content of sodium in a highly concentrated urine of low sodium content with and without addition of sodium chloride. 6 samples of urine of 5 ml each, and 6 samples of the same urine of 5 ml each + 1 ml sodium chloride solution (containing 4.60 mg sodium per ml) were all analysed. The added amount of sodium was determined to 4.47, 4.53, 4.39, 4.59, 4.55 and 4.49 mg sodium. Hence a loss of about 2 per cent occurred during the determination, presumably due to the ashing process. By direct precipitation from a sodium chloride solution no loss occurred.

The same series of determinations was carried out with the addition of 0.92 mg sodium, and for the added amount of sodium we obtained the values 0.90, 0.87, 0.89, 0.90, 0.88 and 0.93 mg.

The presence of this error is much to be regretted, but compared to the variations in sodium content observed in the course of the pres-

ent investigation the error is very small. Moreover, it looks as if the loss, expressed in per cent, does not become appreciably higher at low sodium concentrations.

Determination of potassium. Potassium was precipitated in the form of potassium sodium cobalti nitrite according to the method of PIPER (1940). Since the potassium content of our samples was never above 1 mg it was sufficient to use only 1/5 of the amounts of the precipitation reagents prescribed by this author. The precipitate was washed five times with 3 ml of a saturated aqueous solution of potassium sodium cobalti nitrite.

After washing the precipitate was oxidized with 10 ml 0.02 n ceric sulphate, as was suggested by RAPPAPORT (1933). Ceric sulphate has the advantage, in contrast to permanganate, that it is not reduced spontaneously by heating.

The excess ceric sulphate was titrated with a 0.02 n solution of ammonium ferrosulphate with phenanthroline as indicator.

The potassium content of the samples was calculated from the formula

$\text{mg K} = f \times (\text{ml ceric sulphate consumed by the oxidation} - \text{blank}).$

In 10 determinations on 0.356, 0.593, 0.830 and 1.185 mg potassium the factor f was determined to respectively $0.0645 \pm 4.2\%$, $0.0646 \pm 2.9\%$, $0.0657 \pm 3.1\%$ and $0.0663 \pm 2.0\%$.

By using the average value f 0.0653 and by keeping the potassium content in the samples between 0.35 and 1.2 mg, the error thereby introduced into the determinations was at most 1.5 per cent.

Determination of calcium in urine. This was carried out by precipitating the calcium in 5 or 10 ml urine by means of oxalate and titrating with ceric sulphate as described by LARSON and GREENBERG (1938).

Determination of magnesium in urine. This was carried out on the filtrate from the precipitation of calcium by precipitating magnesium by means of oxyquinoline and weighing the precipitate as described by BERG (1926). Since we were working with precipitates weighing as little as 8 mg the error in this determination is rather large. By determinations of magnesium in a magnesium calcium solution with a content of magnesium corresponding to a precipitate of magnesium quinolate of 8 mg, the error was as large as 10 per cent.

Determination of pH in urine. The hydrogen ion concentration was measured by means of a glass electrode at $20^\circ \pm 2^\circ$. Since the samples were highly concentrated and the pH did not exceed 5.75 no precautions were taken to prevent the diffusion of carbon dioxide.

Experimental Conditions.

The experimental subjects were young men about 20 years of age. By ingestion of ammonium chloride the subjects were kept in acidosis for periods of about 14 days, aiming at a total CO_2 -concentration in serum of ca. 18 milliequivalents.

As was mentioned in a previous paper the formation of ammonia in the beginning of such an acidosis is small, but increases gradually

and reaches its maximum value in the course of ca. 4 days (RYBERG 1948 a).

Experiments were therefore carried out both in the beginning and at the end of the acidosis in order to be able to compare the conditions both when the formation of ammonia is small and when it is large.

From 19.00 the evening before an experiment and until the experiment terminated at ca. 17.00 the following day the subject got neither food nor water in any form. This was done in order to obtain small values of the diuresis and the salt excretion on the day of the experiment. No ammonium chloride was ingested during this period, since experiments showed that then a further reduction in the excretion of water and salts was obtained.

It must be assumed that when the diuresis is very small the urine has its maximum concentration of salts, and a further reduction of the diuresis will then entail a reduction in the amount of positive ions which per unit time reaches the distal part of the tubule. It was therefore considered possible, by the technique just described, to produce such a deficiency in positive ions in the tubular liquid that this would entail a decrease in hydrogen ion concentration and in the formation of ammonia, granted the correctness of the assumption that NH_4^+ and H^+ are exchanged with cations from the urine.

Samples of urine were collected every 1.5 hour in the course of the day of the experiment and pH, diuresis per hour and the excretion of Na^+ , K^+ , Mg^{++} and Ca^{++} were determined. In some experiments a quantity of water, containing either NaCl or KCl , was ingested, in order to investigate the effect of offering the cells of the tubuli a new ample supply of cations. Since the diuresis then increased these samples could be collected every 45. minute.

Results.

Table 1 gives the results from 3 days during the same period of acidosis.

It will be seen that in all the experiments the total concentration of positive ions is remarkably constant, and it is difficult to explain this constancy otherwise than by assuming that the urine, under the conditions of these experiments, has reached its maximum concentration of ions.

On the second day during the acidosis (Table 1), when the formation of ammonia is still only relatively little increased, it depends only to a small extent on the diuresis, while on the 6. and 10. day the formation of ammonia depends strongly on the diuresis. The concentration of ammonia in the urine does not exceed 320 millimoles per liter, and when this value is reached the forma-

Table 1.

Exp. Subj. P K.

Day during acidosis	Total concurrent time in min.	Diuresis per hour in ml	pH in urine at 20° ± 2°	NH ₃ in millimoles per liter urine	Na in millimoles per liter urine	K in millimoles per liter urine	Ca in millimoles per liter urine	Mg in millimoles per liter urine	Total amount of positive ions in millimoles per liter urine	NH ₃ -excretion in millimoles per hour	Na-excretion in millimoles per hour	K-excretion in millimoles per hour
2	0—90	30	5.12	191	75	83	12.2	6.1	367	5.75	2.25	2.49
	90—180	22	5.26	231	54	71	10.3	5.9	372	5.08	1.18	1.56
	180—270	19	5.33	248	43	66	9.6	5.7	372	4.72	0.81	1.26
	270—360	16.5	5.40	300	16.9	41	12.2	8.5	379	4.95	0.28	0.67
	360—450	18	5.39	265	24.4	36	14.4	10.3	350	4.76	0.44	0.65
	Subject drinks 300 ml of 4 % of NaCl Solution											
6	510—555	40.5	4.81	132	143	49	12.9	7.9	345	5.35	5.8	1.96
	555—600	42	4.75	126	147	52	10.0	4.1	339	5.28	6.2	2.19
	0—90	30	5.49	284	20.3	49	8.1	5.7	367	8.5	0.61	1.47
	90—180	22	5.66	323	3.0	52	8.2	5.5	392	7.1	0.066	1.14
	180—270	22.5	5.67	322	3.8	41	9.3	5.7	382	6.6	0.086	0.92
	270—360	20	5.67	320	4.1	47	8.4	5.2	385	6.4	0.081	0.94
10	360—450	18	5.63	324	4.6	37	10.9	5.3	382	5.8	0.083	0.66
	Subject drinks 300 ml of 2 % KCl Solution											
	510—555	74.5	5.00	124	71	159	6.1	2.8	363	9.2	5.3	11.9
	555—600	58.5	5.01	153	54	144	7.4	3.8	362	9.0	3.1	8.4
	0—90	31	5.43	278	25.4	34	19.6	5.3	362	8.6	0.78	1.05
	90—180	25.5	5.50	309	10.7	32	23.4	8.9	384	7.9	0.27	0.81
10	180—270	20.5	5.71	315	3.8	28	19.2	6.1	372	6.5	0.078	0.57
	270—360	19	5.74	313	3.1	27	20.6	7.1	371	6.0	0.059	0.51
	360—450	20	5.74	311	3.0	32	15.8	5.3	367	6.2	0.060	0.63
	Subject drinks 300 ml of 4 % NaCl Solution											
	510—555	43.5	5.05	210	79	45	11.3	4.5	350	9.1	3.5	1.98
	555—600	46	5.03	194	82	51	10.5	3.9	341	9.0	3.8	2.35

tion of ammonia per hour decreases proportionally with the diuresis.

On days with a large formation of ammonia the excreted amount of sodium decreases to about 1/10, while the diuresis is reduced only to about 2/3. It is a very striking fact that the excretion of sodium is reduced to less than 1/10 millimoles per hour and the concentration of sodium to ca. 4 millimoles per liter.

The excretion of potassium is also considerably reduced, but the concentration in the urine never reaches such a low level as is the case with sodium.

The concentration of calcium and magnesium does not seem to depend much on the diuresis, while the excretion per hour conse-

quently on the whole must remain proportional to the diuresis. However, the variations in the excretion of these substances are so large, that we can only infer that magnesium and calcium do not show the same behaviour as sodium.

On the 10. day the subject received at the end of the experiment 300 ml of a 4 per cent NaCl solution per os. The formation of ammonia per hour thereby increased to values which were higher than those found at the beginning of the experiment.

On the 6. day the subject received 300 ml of a 2 per cent solution of KCl, and the effect on the ammonia formation was principally the same as after ingestion of NaCl. BUNGE (1873) has demonstrated that the ingestion of potassium entails an increased excretion not only of potassium but also of sodium, and in the experiment under consideration we observed a very large increase in the sodium excretion. Ingestion of sodium also caused an increased excretion of potassium.

The results mentioned above might be interpreted to indicate that NH_4^+ is transferred to the tubular liquid by exchange with sodium ions. Granted this assumption the very large reduction in sodium excretion and the simultaneous reduction in the formation of ammonia might be explained by assuming that a point had been reached where almost the whole quantity of sodium reaching the distal tubules was exchanged with ammonia.

However, one might also imagine that the organism always reacts to a corresponding water and salt deficiency by such a large reabsorption of sodium; the reduction in the formation of ammonia may be due to its dependence on the diuresis.

This possibility can be checked by a consideration of the experiment of the 2. day. The conditions are here quite similar to those of the 6. and the 10. days, with the sole exception that the acidosis has been of shorter duration, and that therefore the formation of ammonia is smaller. The reduction in the excretion of sodium is here much smaller, in spite of the fact that the diuresis does decrease to the same extent as in the other experiments. Further, the formation of NH_4^+ is not so strongly affected. After the ingestion of 300 ml of a 4 per cent NaCl solution there is no appreciable increase in the formation of ammonia.

Table 2 shows the results of 3 experiments carried out on another subject during one and the same period of acidosis. The excretion of ammonia here has all the features characteristic of the experiments reported in table 1.

Table 2.
Exp. Subj S.J.

Day during acidosis	Total concurrent time in min.	Diuresis per hour in ml	pH in urine at 20° ± 2°	NH ₃ in millimoles per liter urine	Na in millimoles per liter urine	K in millimoles per liter urine	Ca in millimoles per liter urine	Mg in millimoles per liter urine	Total amount of positive ions in millimoles per liter urine	NH ₃ -excretion in millimoles per hour	Na-excretion in millimoles per hour	K-excretion in millimoles per hour
2	0—90	40	4.98	133	78	125	14.0	6.5	357	5.3	3.12	5.0
	90—180	34.5	4.98	156	84	102	13.3	5.5	361	5.4	2.91	3.5
	180—270	32	5.04	150	63	107	15.1	7.0	342	4.8	2.02	3.4
	270—360	26	5.05	187	61	85	12.7	5.2	351	4.9	1.60	2.22
	360—450	24.5	5.11	189	66	89	13.1	4.8	362	4.6	1.62	2.18
5	0—90	26.5	5.74	319	5.3	21.2	12.0	5.0	363	8.5	0.140	0.56
	90—180	29	5.50	281	21.0	27.6	12.4	4.8	347	8.2	0.61	0.80
	180—270	34.5	5.36	245	77	31.0	14.2	6.2	373	8.5	2.65	1.07
	270—360	31.5	5.44	252	61	34.3	9.3	3.4	360	7.9	1.91	1.08
	360—450	26	5.50	273	26.1	37.5	10.3	5.2	352	7.1	0.68	0.98
	450—540	19.5	5.71	322	3.7	34.7	8.9	4.6	374	6.3	0.072	0.68
10	540—630	19	5.75	317	3.3	27.1	11.7	6.0	365	6.0	0.063	0.52
	0—90	36	5.57	262	6.9	36	12.3	5.4	323	9.4	0.25	1.29
	90—180	33	5.43	260	46.2	24	10.6	3.8	345	8.6	1.52	0.80
	180—270	30	5.53	283	12.5	21	13.5	5.7	336	8.5	0.37	0.64
	270—360	32.5	5.36	263	58	33	11.5	4.7	370	8.5	1.90	1.07
	360—450	35.5	5.24	248	74	33	9.5	4.1	369	8.8	2.61	1.16
	450—540	22	5.61	314	15.0	19	11.4	3.9	363	6.9	0.33	0.43
	540—630	17.5	5.73	333	3.7	19	12.1	4.6	372	5.8	0.065	0.33
Subject drinks 400 ml of 4 % NaCl Solution												
	690—735	43.5	5.08	214	75	44	10.6	5.5	349	9.3	3.2	1.90
	735—780	44.5	5.03	211	78	47	9.8	4.6	350	9.4	3.5	2.07

As to the excretion of H⁺ it will be seen that the hydrogen ion concentration varies with the content of sodium in the urine. In the experiments where sodium or potassium is administered a considerable decrease in pH is seen in spite of the fact that the diuresis increases. There is, however, a certain difference in the behaviour of NH₄⁺ and H⁺. On the 2. day in table 1, where the excretion of NH₄⁺ is not large and there is accordingly no considerable sodium deficiency, pH nevertheless decreases to very low values when Na⁺ is ingested. This seems to indicate that while the excretion of ammonia is most sensitive to variations in the sodium content when this is very low, pH will depend on the Na⁺-excretion over a wider range of concentration.

The experiments on the 5. and 10. day, table 2, point in the same direction. Here the various changes in the content of sodium

during the beginning of the experiments are accompanied by corresponding variations in pH while the excretion of NH_4^+ does not seem to be influenced by the changes.

Discussion.

The experiments show that pH of the urine and the excretion of ammonia depend on the amount of cation reaching the distal tubules per unit time. It is most natural to explain this by assuming that H^+ and NH_4^+ are excreted by exchange with cations from the tubular fluid, which is tantamount to saying that the anions formed together with H^+ and NH_4^+ in the cells are not excreted in the urine.

The experiments further show, however, that Na^+ occupies a privileged position among the positive ions of the tubular fluid. It is true that an ingestion of potassium during a cation deficiency in the urine can bring about an increase in the formation of ammonia, just like an ingestion of sodium, but there is nevertheless the decisive difference between the two metals that the concentration of potassium never sinks to very low values, while on the contrary the exchange process seems to require practically all the sodium present when a deficiency in cations exists.

In this connection it must be remembered that the process not only consists in an exchange of NH_4^+ and H^+ with cations from the urine. The next step must consist in the transfer of these cations to the interstitial fluid.

It is now possible that the sodium ion owes its privileged position to the circumstance that Na^+ is transported actively to the interstitial fluid.

An active transport of sodium has been assumed by KROGH (1946) to take place in muscle cells and CONWAY, FITZGERALD and MACDOUGALD (1946) have been able to demonstrate directly an active extrusion of Na^+ , sensitive to cyanide poisoning, from the cells of the distal part of the frog's tubules in vitro.

According to PITTS and ALEXANDER (1945) the source of the hydrogen ions delivered to the tubular fluid is carbon dioxide formed in the cells or diffused into them from the blood. Their reasons for this assumption is that H_2CO_3 is the only acid available in sufficient quantities and that the formation of acid in their experiments turned out to be sensitive to sulfanilamide, which inhibits the effect of carbonic anhydrase. They did not

succeed in blocking the formation of acid completely, and it is impossible to tell to what degree the excretion of H^+ has been paralysed in their experiments, as some of the hydrogen ions may have been spent in transforming HCO_3^- in the tubular liquid to CO_2 , which has then diffused back to the organism. Even the pH of about 7.3 in the urine obtained in two experiments where the acidosis was not too heavy, is by no means conclusive evidence that no hydrogen ions have been delivered to the urine (cf. PITTS and LOTSPEICH (1946) and RYBERG (1948)).

It is impossible to know if the deficient inhibition in the experiments is due to the sulfanilamide not completely preventing the transformation of CO_2 to H_2CO_3 or if other acids, for instance lactic acid, are taking part in the formation of hydrogen ions too.

If it be true that the sources of the hydrogen ions are acids formed in the cells, the neutrality regulation can only be effective if the anions corresponding to the acids are transferred to the organism and take up hydrogen ions during their combustion or — when the question is of HCO_3^- — during ventilation in the lungs.

It has been mentioned that in all probability an active transport of Na^+ takes place from the cells to the interstitial fluid. This process will give rise to a potential difference, which will not only favour the transport of anions from the cell to the interstitial fluid but also tend to block the passage of H^+ and NH_4^+ in this direction. If the anions of the urine are able to invade the cells, their further passage to the blood will also be accelerated by the active transport of Na^+ and will eventually be able to take place against a concentration gradient. Finally a tendency will be created for K^+ and H^+ to migrate from the interstitial fluid to the cell. If a transport of K^+ may in fact take place through the cells from blood to urine cannot be known, but it cannot be considered quite improbable, as LIANG (1929) has been able to show that K^+ can diffuse in this direction through the cells of the frog's tubule. Under the conditions of my experiments the concentration of potassium was always much larger in urine than in plasma.

Without knowing both the concentration of the ions and their relative velocity of diffusion through the different parts of the cell it is impossible to get a theoretically founded conception when it comes to the question about which of the mentioned possible transports of ions are the predominant ones. The matter will be further complicated if other active processes apart from the

transport of Na^+ are taking place, which seems quite probable. It can be stated, however, that the rate of permeability between the different ions may well make it possible to excrete H^+ and NH_4^+ through the luminal membrane against a concentration gradient, even if no active processes are assumed to take place here.

If the concentration of potassium is very high in the urine, K^+ may diffuse through the cells against the potential difference. In that case it may be unnecessary to transport the usual amount of Na^+ to the interstitial fluid, partly because K^+ may be exchanged with H^+ and NH_4^+ just like Na^+ , and partly because the part of the transport of sodium can be saved, which has previously corresponded to the flow of potassium from interstitial fluid to tubular liquid. The experiments show that ingestion of potassium will in fact be accompanied by a larger excretion of sodium, but of course this can also be explained by assuming that more sodium will under these circumstances reach the distal tubules.

It will be seen that if our general concept of the processes involved in the neutrality regulation is correct, *i. e.* that H^+ and NH_4^+ are exchanged with cations from the urine while the corresponding anions migrate to the interstitial fluid together with these cations, then an active transport of the sodium ion may be essential to the removal of H^+ , NH_4^+ and their anions from the cell and consequently to the rate of formation of these ions.

Summary.

The aim of the present work has been to investigate whether the urinary excretion of NH_4^+ and H^+ takes place by an exchange with positive ions from the urine.

pH in the urine and the excretion of NH_4^+ were investigated under conditions, where the amount of positive ions reaching the distal part of the tubules was reduced. The following results were found:

1. At high values of the excretion of NH_4^+ , a cation deficiency resulted in a decrease in this quantity.
2. Simultaneously a very large reduction in the urinary excretion of Na^+ and a more moderate decrease in the excretion of the other cations were found.
3. The pronounced reduction in the urinary excretion of Na^+ was absent in the beginning of an acidosis, when the formation of ammonia was small.

4. The hydrogen ion concentration of the urine varied with the content of Na^+ over a considerable range of concentration. When Na^+ or K^+ were ingested, pH decreased in spite of the increasing diuresis.

The experiments support the idea that NH_4^+ and H^+ are excreted by exchange with cations from the tubular fluid. It is emphasized that the privileged position apparently occupied by the sodium ion is not necessarily associated with the exchange process itself but may be due to an active transport of this ion from the tubular cells to the interstitial fluid.

The possible effects of an active transport of sodium on the migration of other ions are discussed.

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Pharmacological Properties of Sorbide Dinitrate.

By

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Among drugs, used in the therapy of angina pectoris and hypertension, amyl nitrite, glyceryl trinitrate, erythritol tetranitrate and mannitol hexanitrate were studied pharmacologically and clinically already 50 years ago, *e. g.* by BRADBURY in 1895 and by MARSHALL in 1897.

The search after vasodilating drugs was actualized during the last decade. KRANTZ and coworkers investigated the effect of a series of alkyl nitrites and nitrates (KRANTZ, CARR, FORMAN and ELLIS 1938 a, b). Among other compounds they synthesized seven hexitol anhydrides and their nitrates (KRANTZ, CARR, FORMAN and ELLIS, 1939 a), *i. a.* mannide and sorbide dinitrate. The pharmacology of mannide dinitrate was more closely studied in animals, especially the effect on coronary circulation and on blood pressure in dog, and some experiments were performed on healthy subjects and in hypertensive cases (KRANTZ, CARR, FORMAN and ELLIS 1939 b). The compound was suggested to be useful in the treatment of hypertension and angina pectoris, but so far no report on its clinical use has appeared.

As to the mechanism of action of nitrites and nitrates, KRANTZ and coworkers attribute an increase in depressor response to a lesser degree of water solubility and to a corresponding greater degree of oil solubility (KRANTZ, CARR and FORMAN 1939 c), and

made probable that the action of organic nitrates was dependent upon the unhydrolyzed molecule (KRANTZ, CARR, FORMAN and CONE 1940). RATH and KRANTZ (1942 a) worked out a method for the quantitative determination of nitrite in blood in different species and were able to show that intravenous or oral administration of organic nitrates in dogs did not increase the blood-nitrite content (1942 b). Finally RATH and KRANTZ (1942 c) demonstrated that sodium nitrite reduced the arterial blood pressure of unanesthetized hypertensive rats.

Other compounds, too, have been studied. BJERLÖV (1943) reported the effect of pentaerythritol-tetranitrate in 10 cases of hypertension and in 26 cases of angina pectoris. FLODMARK and WRAMNER (1943) made a study of the effect of mannitol hexanitrate in 19 healthy subjects and in 9 cases of hypertension. These compounds have been clinically used.

None of these preparations seemed, however, to equal the effect of erythritol tetranitrate, and the problem of an equivalent was still open.

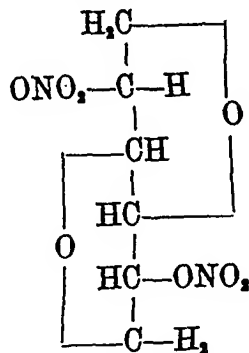
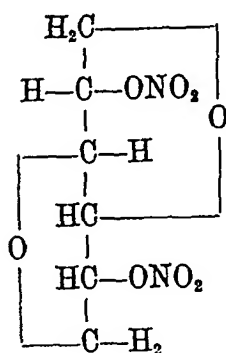
Regarding one isomere of mannide dinitrate, viz. sorbide dinitrate, so far only some preliminary experiments showing its depressant action on the healthy dog's blood pressure were performed by KRANTZ and coworkers (KRANTZ, CARR, FORMAN and ELLIS 1939 b), but no other studies were made. It was therefore thought worthwhile to examine sorbide dinitrate more closely.

The aim of this work is to study the effect of sorbide dinitrate (SDN) on toxicity, blood pressure, respiration, coronary circulation and isolated gut in animals in comparison to mannide dinitrate (MDN) and to establish its action in man in hypertensive subjects.

A. Physico-Chemical Properties.

Sorbide dinitrate (SDN), $C_8H_{16}O_2(ONO_2)_2$, mol. w. 236.1, is a white, crystalline compound, stable in air, melting point $70^\circ C$, optical rotation $(\alpha) 20^\circ + 134^\circ$ ($c = 1.0$ in alcohol); solubility in water 1—2 : 1,000, freely soluble in alcohol and ether, and in arachis oil to about 50—100 : 1,000 at $50^\circ C$. Mannide dinitrate (MDN), see FORMAN, CARR and KRANTZ (1941)¹.

¹ I am indebted to Kärnbolaget, Stockholm, for synthesizing the compounds and placing larger amounts of SDN (Harrical) and MDN at my disposal.



1, 4, 3, 6-dianhydro-sorbitol-
2,5-dinitrate (SDN)

1, 4, 3, 6-dianhydro-mannitol-
2,5-dinitrate (MDN)

B. Toxicity.

1. Oral Administration.

Single dose: A number of white mice, weight 10—25·g, were fed with a single dose of 1.5—10 mg/g SDN and MDN resp., the drug being mixed with the food.

1—2 mg/g brought about only a slight reaction: the animals lost their spontaneous activity and sat, their back curved.

3—5 mg/g caused ataxia, excitation with running movements, the Straubtail-phenomenon, the hair standing out from the body, eventually convulsions and loss of righting reflexes. Some animals succumbed on this dose, others on 5—10 mg/g.

Repeated doses:

1) *SDN:* 3—4 mg/g per day fed for 2 days led to convulsions; 2 mg/g/day for 3 days had no lethal effects, whereas 3—6 mg/g/day for 3 days were fatal.

2) *MDN:* 6 mg/g fed for two days led to convulsions. 2 mg/g/day fed for three days had no lethal effect, whereas the animals succumbed on 3—6 mg/day fed for three days.

2. Subcutaneous Administration.

A. White Mice.

Due to the slight solubility in water, the substances SDN and MDN were solved in arachis oil to 10 % concentration and kept at 50° C, SDN having a higher solubility, and only slowly crystallizing at room temperature, MDN crystallizing rather rapidly when kept at room temperature. The acute toxicity was established in two series of experi-

ments, one with high doses, causing 100 % mortality, for establishing median time to convulsion and median time to death (I), and a second series with doses, giving a varying response, for determination of median convulsive dose and median lethal doses (II).

Series I. Time-Response.

SDN: 0.033 ml and 0.050 ml per g mouse were injected subcutaneously of a 10 % solution; *MDN*: 0.033 ml/g subcutaneously, concentration 10 %, 15 mice being used for each dosage and each compound.

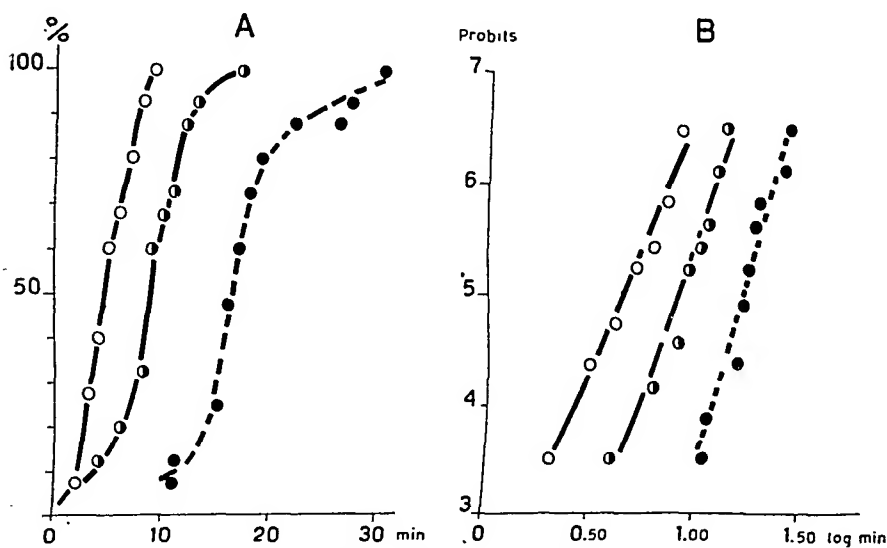


Fig. 1. Time to Appearance of Convulsions.

- A. Percentage, plotted against time in minutes.
B. Probits, plotted against log time in minutes.

○ — 0.035 mg/g SDN
◐ — 0.050 mg/g SDN
● — 0.033 mg/g MDN

The number of animals, showing the same time to convulsion was transformed to percent of the total number of animals, these percentages added, and plotted against time in minutes (fig. 1 A). The median convulsion time (CT_{50}) is the intersection of the time-response curve and 50 % response.

A picture of the relation between dosage and time and the different compounds, more apt for quantitative work, is given in fig. 1 B. The percentage of animals, showing the same time, were added, the added percentage transformed to probits (BLISS 1938), and the probit values plotted against log time in minutes. The curves now became approximately rectilinear, which implies

the log times to be approximately normally distributed. The median time was graphically computed from the intersection of the straight log time-response line and probit 5 corresponding to the 50 % value.

In the same way the median time to death was determined by plotting the added percentages transformed to probits against log time. The median lethal times (LT_{50}) and their standard deviations are given in table 1.

Table 1.

Time to Convulsion (CT_{50}) and to Death (LT_{50}) in Mice.

	Sorbide dinitrate (SDN)		Mannide dinitrate (MDN)
Dose: ml/g	0.033	0.050	0.033
mg/g	3.3	5.0	3.3
	I. Time to convulsion (CT_{50}).		
Median Time (CT_{50})	8.1 min	4.4 min	16.2 min
Stand. dev. (σ)	39 % (3.1 min)	48 % (2.1 min)	32 % (5.1 min)
Number of animals	15	15	15
	II. Time to Death (LT_{50}).		
Median Time (CT_{50})	103 min	81 min	182 min
Stand. dev. (σ)	53 % (54 min)	41 % (33 min)	56 % (102 min)
Number of animals	15	15	15

It is seen from the table that the effect of SDN, when given in the same dose as MDN, occurs in half the time of MDN; likewise an increase in dosage brings about a shortening of the times.

The slopes (b) of the curves are nearly parallel to each other, and the difference between compounds and doses is merely a shift to the left or right. The slopes of these curves are the inverse value of the standard deviation (σ) ($\sigma = \frac{1}{b}$), and the standard deviations (σ) are thus of the same magnitude.

This is quite natural for two doses of one and the same substance, the standard deviation σ being a characteristic of the compound and the animals used for assay. Thus two different doses of one and the same compounds administered on the same stock of animals, will give the same standard deviation. On the

other hand, the approximate agreement of the standard deviations of the two compounds SDN and MDN is an expression of the close relationship between the two isomeres, also in their biological action.

Series II. Dose-Response.

The following doses of a 10 % solution by weight in arachis oil of SDN and MDN resp. were injected into groups of 10–15 white mice, weighing 9–18 g.

SDN: 0.4, 0.6, 1.0, 1.5 and 3.3 mg/g; and of MDN: 0.7, 1.0, 1.5, 2.25 and 3.3 mg/g. The percentage of animals within each group having convulsions and succumbing resp. was noted and plotted against dose in mg/g in figure 2 A. The record shows the typical dose-response curve for the lethal doses.

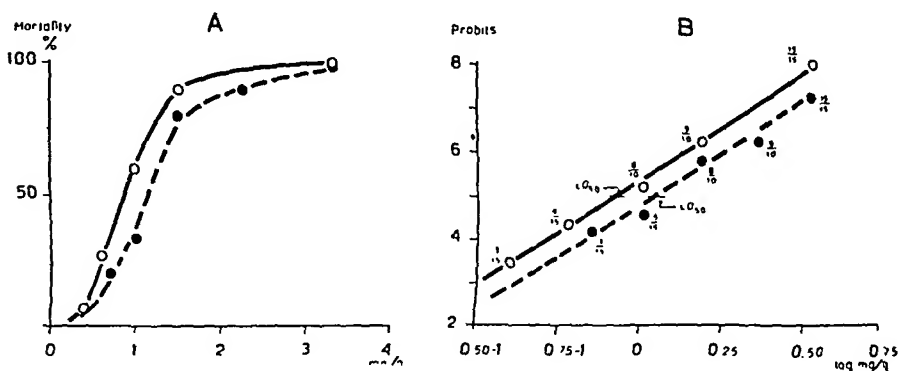


Fig. 2. Lethal Dose in Mice.

A. Percentage plotted against dose.

B. Probits plotted against log dose.

○ — ○ SDN 10 %
● — ● MDN 10 %

In figure 2 B the percentages are transformed to probits (BLISS 1938), and plotted against log dose. This gives a rectilinear curve for both compounds, which implies log doses to be normally distributed. The parallel course of the curves implies the same standard deviation, which, as stressed before, is one expression of the close relationship between the two substances, not only chemically but also biologically. The lethal doses (LD_{50}) were established from the curves. In the same way the convulsive dose (CD_{50}) causing 50 % response, was established from similar curves for the percentage of animals, reacting with convulsions (table 2).

It is likely that in white mice SDN has a slightly larger toxicity and a somewhat stronger convulsive action than MDN.

Table 2.
Convulsion (CD_{50}) and Lethal Doses (LD_{50}) in Mice.

	Sorbide dinitrate SDN	Mannide dinitrate MDN
Convulsion Dose: CD_{50}	0.24 g/kg	0.78 g/kg
Stand. dev. (σ)	60 %	82 %
Number of animals	65	65
Lethal Dose: LD_{50}	0.84 g/kg	1.12 g/kg
Stand. dev. (σ)	47 %	46 %
Number of animals	65	65

b. Rats.

Experiments were also performed on white rats, weighing 150—270 g. 3—5 groups of animals were injected with a 10 % solution in arachis oil of SDN and MDN resp., 52 animals in all.

SDN: 0.5, 0.8 and 1.2 mg/g. MDN: 0.5, 0.8, 1.2 and 1.6 mg/g.

The animals succumbing died within 6—8 hours and showed no definite excitation, only a few showing convulsions before exitus; loss of righting reflexes occurred only late in the sequence of symptoms. Death was due to respiratory and cardiac paralysis, LD_{50} being 0.77 g/kg for SDN and 0.74 g/kg for MDN, thus no difference.

3. Histological Examination.

Some twenty animals were examined histologically. One part of the animals were fed orally with 2—4 mg/day of SDN, resp. MDN, for 3 days and then sacrificed. Another part was given 0.7 and 1 mg subcutaneously in 10 % solution in one dose of each compound and sacrificed after 3 days. A third part of animals served as controls. No pathological changes were seen in the brain, heart, liver, spleen, kidney or suprarenal glands in either of the groups.¹

C. Effect on Blood Pressure and Respiration.

The effect on blood pressure and respiration was tested in rabbits, anesthetized with 1.75 g urethan per kg intravenously in a concentration of 20 %, trachea and one jugular vein being cannulated and the arterial blood pressure recorded from one carotid artery by means of a mercury manometer. The respiration was quantitatively recorded by means of a body plethysmograph, according to EULER and LILJESTRAND (1936).

¹ The author is indebted to G. AURELL, M. D., Dept. of Histology, Caroline Inst. for examination of the slides.

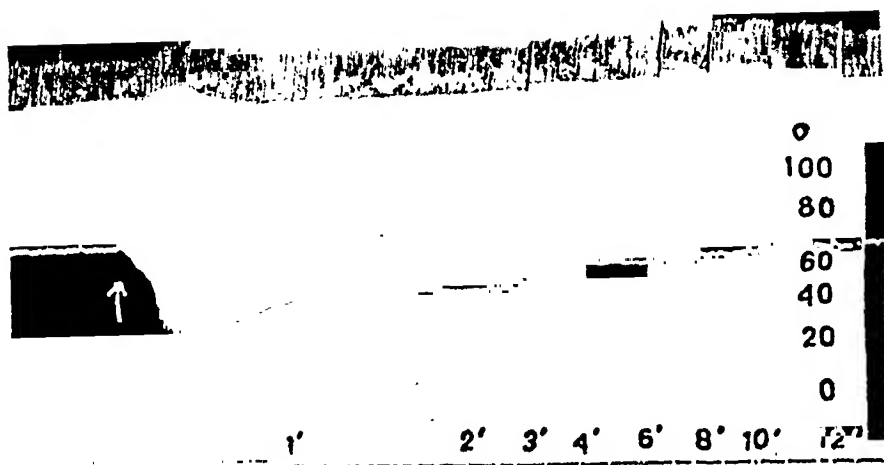


Fig. 4. Effect of SDN on Respiration and Blood Pressure in Rabbit.

Injection of 0.15 mg SDN/kg in 0.1 % solution (in saline).

Blood Pressure (middle curve): Maximal fall: 34 mm Hg = 47 % of basal level. Duration: 12 min.

Respiration (upper curve): Increase in rate +8.8 %, in depth +20 %, in ventilation +30 %.

Time: 10 seconds.

action on blood pressure of a rather long duration. SDN is more active than MDN, the dose of MDN having to be increased by 30—50 % in order to give the same response as that of SDN.

D. Perfusion of the Isolated Heart.

A number of experiments were performed in order to study the action of SDN on the coronary circulation, in relation to that of MDN.

Method: A Langendorff preparation of the isolated rabbits heart was used, perfused with a 0.0075—0.0125 % solution of SDN and MDN resp. using Tyrode solution as control. The solutions were kept in bottles with Mariottes tubes, aerated with 5 % CO₂ + 95 % O₂ and warmed to 37° C; the perfusion pressure was kept constant at 80—100 cm of water. The experiments started with perfusion of Tyrode solution for 5—10 minutes. Then the perfusion was switched over to SDN or MDN resp. for 5 minutes, then Tyrode again and so continuing perfusing the heart with Tyrode and test solution alternately. In this way it was possible in one and the same test to compare a compound with the effect of Tyrode, and the influence of the continuous decrease in vitality of the heart was eliminated.

The amount of fluid perfused per min. was taken as a quantitative measure of the coronary circulation. The amount was measured separately for each minute during the last 2 or 3 minutes of each period,

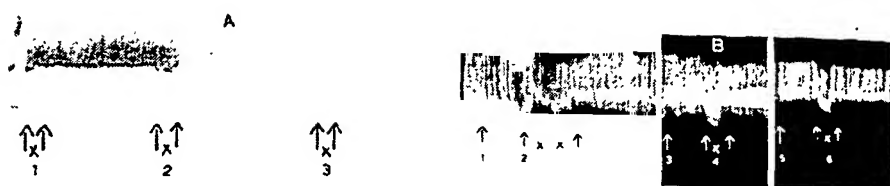


Fig. 5. Perfusion of Isolated Heart (Langendorff Preparation): A. SDN, B. MDN.

A. 1. 0.0125 % SDN in saline. 2. Tyrode. 3. 0.0125 % SDN.

B. 1. Tyrode *Flow: 6.5 ml/min. Time from start: 35 min*
 2. 0.0125 % MDN » 9 » » » 42 »
 3. Tyrode » 6 » » » 59 »
 4. 0.0125 % MDN » 8 » » » 68 »
 5. Tyrode » 5 » » » 84 »
 6. 0.0125 % MDN » 8.5 » » » 97 »

× × = Washing.

Time: 10 seconds.

the mean of these 1-minute flows being used as the ultimate value and expressed in per cent of the arithmetical average of the preceding and the following control. In some experiments the heart could be kept going for 3—4 hours, allowing about 30—40 periods of flow to be recorded and measured.

The beats of the ventricles, in some experiments of the auricles too, were recorded on a kymograph.

A typical record is given in figure 5 A, showing the increase in the magnitude of the beats, due to SDN perfusion. In fig. 5 B MDN was perfused, and the record shows the increase of flow, after MDN, in spite of the continuous diminishment of the beats, due to decrease of viability of the heart.

A number of experiments were performed with varying concentrations of SDN and MDN, 0.0075—0.0200 %, 123 tests on 10 hearts with SDN, 64 tests on 8 hearts with MDN, and 37 tests with both compounds in 3 hearts. The result was that SDN had a threshold concentration of 0.0075 % on an average against 0.0100—0.0125 % for MDN; the threshold concentration brought about an increase in flow of 30—40 %, and higher concentrations a higher increase in flow.

It can thus be concluded that SDN and MDN have a specific action on the isolated, perfused Langendorff heart, invariably giving an increase in coronary flow, when perfused in a concentration over a certain threshold, SDN having a lower threshold concentration, and a somewhat higher activity.

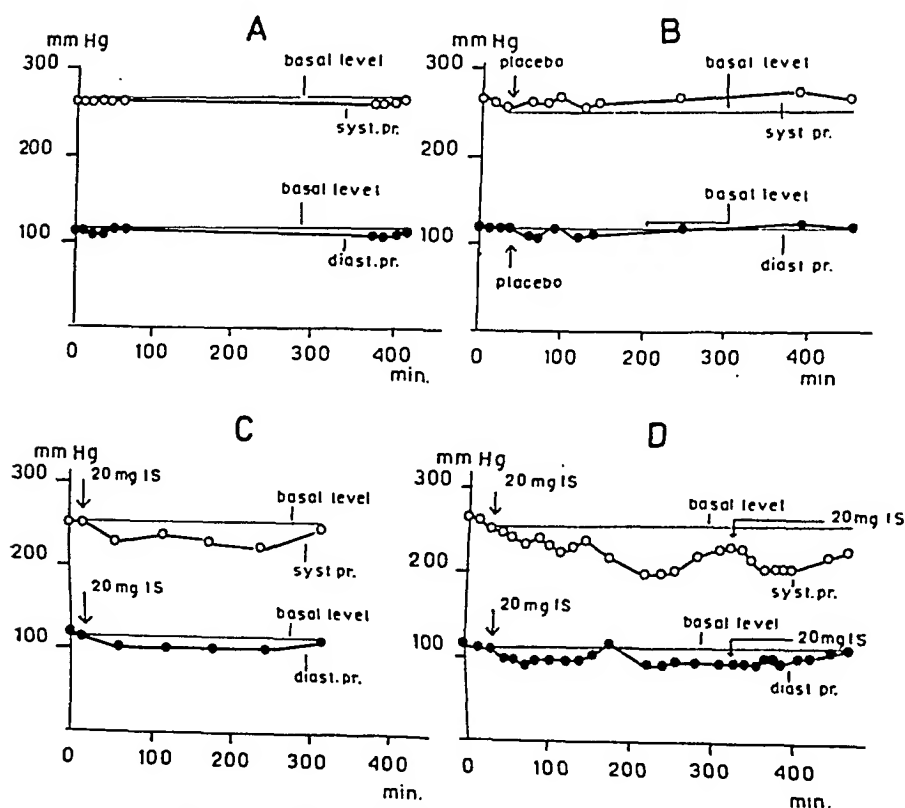


Fig. 6. Effect of SDN on Hypertension in Man.

- A. Blind test, no medication.
- B. Blind test, placebo given.
- C. 20 mg SDN.
- D. 2×20 mg SDN. (IS = SDN.)

E. Isolated Gut.

Some tentative experiments were made on the isolated rabbit's ileum.

The effect was a transient or definite decrease of tone and diminution of amplitude, SDN and MDN differing quantitatively, SDN giving an effect in lower dose than MDN.

F. Action in Man.

The action of SDN was finally tested in man, in hypertensive subjects.¹ 24 hypertensive hospitalized patients were used as test subjects and in all 72 experiments were performed. The average systolic blood

¹ These experiments were performed together with I. G. PORJÉ, M. D., at that time at the Medical Dept. of Södersjukhuset, Stockholm.

pressure was 210 mm (165—265 mm Hg). All subjects had been confined to bed for 2—3 weeks before the test, during which time their blood pressure had become constant; the subjects had been fasting before the experiment, were kept in bed during the whole experiment, and were not allowed to eat for the first 5—6 hours. All experiments were started in the morning at approximately the same time. SDN was given orally as tablets of 10 mg in a dose of 2 (—4) tablets. Its effect was compared to that of a placebo or blind tablet of the same shape and containing the same constituents but without SDN. In 4 experiments no tablets at all were administered. Each subject was submitted to at least 2 tests, one with the placebo, and one with the SDN tablets. The experiment started with measuring the blood pressure auscultatory every 15 minutes, 3—5 times. When a constant level was reached a tablet was given, neither the test subject, nor the clinician knowing if a placebo or the SDN tablet was given. The blood pressure at the moment of indulging the tablets was denoted as basal value. The blood pressure was followed every 15 min. for 3—5 hrs, and then at longer intervals for up to 18—24 hrs, if possible till the blood pressure had returned to the basal level.

In all 24 placebo tests, 4 blind tests, and 44 SDN tests were carried out on the 24 subjects.

The placebo tablet had no effect whatever on blood pressure in the blind tests, the average change in pressure being — 0.8 mm Hg, with variations from a lowering of 30 mm Hg to an increase of the same amount. This is a confirmation of the constant level of the blood pressure in the patients examined and an expression of the non-suggestibility of these subjects.

The SDN-tablets, as a rule 20 mg being given, gave an average fall in blood pressure of 32.4 mm Hg, corresponding to 15.5 % of the average basal blood pressure.

A typical experiment is illustrated in figure 6 A—D.

In 6 A the blood pressure during a day with no medication (A) is shown to display a slight fall of maximally 10 mm Hg. In B a blind tablet was given, the blood pressure during this experiment increasing by 20 mm Hg. In C a single dose of 2 tablets SDN à 10 mg was administered, causing a fall of 40 mm Hg, lasting for 5 hours. In D a second dose was given after 5 hours and still had a good effect.

Only one subject out of 24 (3.8 %) did not respond to SDN with a fall: his blood pressure showed no change in 2 experiments on two different days. As the blind test in this subject showed a slight increase during the day, no change with SDN might actually mean a slight decrease from the control experiment.

A survey of the results is given in table 3.

Table 3.

Effect of Sorbide Dinitrate on Hypertension in Man.
(44 Experiments in 24 Subjects.)

	Average	Variations
Basal Blood Pressure (Systolic)/ n=44/	210.7 mm Hg	160—270 mm Hg
Maximal Change in Blind Test (Placebo)	— 0.8 mm Hg	—30— +30mmHg
Maximal Change after 20 mg SDN (n = 44)	— 32.4 mm Hg	0—70 mm Hg
Maximal Change after 20 mg SDN (n = 44)	— 15.5 %	0 — -36.8 %
Number of Subjects Reacting with Fall in Blood Pressure	23/24 (96 %)	
Number of Tests with Fall in Pressure	42/44 (96 %)	
Onset of Depressant Action (n = 44)	10 min	3—20 min
Time til Maximal Effect (n = 44) ...	75 min	5—200 min
Duration of Effect (n = 31)	4.2 hours	1—24 hours
Headache: Number of Subjects	10/24 (42 %) ¹	
» » Tests	18/44 (41 %)	

¹ Two of the subjects also got headache on administration of blind tablets.

Among symptoms noted the headache is the principal one, occurring in about 41 % of the cases. The headache seemed to be typical for some subjects, where it appeared also in a second test. Two subjects had headache in the blind test. The headache can be looked upon as an inherent part of nitrite or nitrate medication, as it occurs with all of compounds, belonging to this series. The headache responded well to ordinary analgetics (acetyl-salicylic acid, combined with codein). After medication had been maintained for a certain time the headache vanished as a rule. A means of eliminating the headache was to begin the treatment with subliminal doses, 2½—5 mg, and so gradually to increase the dose till the full effect on blood pressure was obtained.

SDN has further been tested in a series of cases of angina pectoris and claudicatio intermittens.¹ So far it can be said that SDN proved to be of value in cases of hypertension, angina pectoris and claudicatio intermittens.

In a preliminary report PORJÉ (1947) stated that a 1 % ointment of SDN rubbed into the skin, corresponding to a total amount of 20 mg, gave a long-lasting and considerable fall in blood pres-

¹ A comprehensive report of these cases will appear in an appropriate journal. A preliminary report was read before the Swedish Society of Int. Med. in Oct.—45 (GOLDBERG and PORJÉ 1946).

sure in hypertensive cases. Headache occurred only in some few instances, and seemed to occur in those subjects only where the effect on blood pressure was slight or none. No effect on the pulse frequency was seen.

G. Discussion.

The experiments reported establish that SDN and MDN actually lower the blood pressure, increase the coronary circulation and diminish the tone and the amplitude of the gut. SDN has invariably a somewhat stronger action than MDN.

SDN and MDN seem to belong to a group of compounds, where there is a large range from the "therapeutic" to the toxic dose. The effect on blood pressure was caused by 0.1—6 mg/kg as compared to the oral and subcutaneous lethal dose, established in white mice and rat, being 2 g/kg, resp. 0.8—1.1 g/kg. The actual oral doses used in man were 10—20 (—40) mg/70 kg = 0.14—0.30 (—0.57) mg/kg.

When comparing the effects of SDN in hypertensive cases (table 3) with those reported by other workers for other compounds (surveys by GOODMAN and GILMAN 1941 or FLODMARK and WRAMNER 1943), it seems that SDN so far ranks among the most active of the long-acting nitrites and nitrates hitherto used for the treatment of hypertension or angina pectoris with regard to dose, time to onset of action, magnitude of fall in blood pressure and duration of effect.

Summary.

The effect of sorbide dinitrate (SDN) has been examined in animals and man and compared to mannide dinitrate (MDN).

I. Experiments in Animals.

1. The oral and subcutaneous toxicity in white mice and rats is comparatively low, 0.8—1.1 g/kg, being slightly larger for SDN than for MDN in mice but equal in rats.

2. Both compounds lower blood pressure in rabbits in doses of 0.1—6 mg/kg, SDN being more active than MDN and having a slightly larger duration.

3. Both compounds increase the flow through the isolated heart (Langendorff preparation), SDN having a lower threshold concentration (0.0075 %) and a larger effect than MDN.

II. Experiments in Man.

4. SDN tested on 24 hypertensive cases in a dose of 20—(40) mg lowers the systolic blood pressure by 32.4 mm Hg on an average with a duration of 1—24 hours. SDN caused headache in 41 % of the experiments provided that it was not given in a subliminal dose, $2\frac{1}{2}$ —5 mg, and the dose gradually increased, or as a 1 % ointment in a total dose of 20 mg SDN, when no headache occurred.

5. Based on results on patients SDN is suggested to be used therapeutically in cases of hypertension, angina pectoris and claudicatio intermittens and to rank among the most active of the long-acting compounds of this group.

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On the Histaminolytic Activity of Skin Extracts.

By

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In 1929, BEST had succeeded in showing animal tissues to contain a substance capable of *in vitro* inactivating histamine. BEST and MC HENRY (1930) in a more comprehensive paper stated that the new substance was of the nature of an enzyme and proposed for it the designation, histaminase.

In several animals the presence of histaminase has been demonstrated experimentally in various organs. BEST and MCHENRY (1930) noted a particularly powerful histaminolytic activity in the kidney and intestine of the dog, whereas MCHENRY and GAVIN (1932) obtained negative results when working with the kidneys of guinea-pigs and rats respectively. At similar results arrived ROSE, KARADY, and BROWNE (1940). As regards the occurrence of the enzyme within the human system, its presence was demonstrated principally in the kidney, adrenal body, and liver.

The results hitherto on record reveal that an enzyme with lytic action on beta-imidazolethyl-amine is present in organs of various animal species, but that the amount of this substance is subject to large variations from one species to the other.

The question then arises of whether histaminase is capable of acting not only under the ideal conditions obtaining *in vitro* but within the living system. There is some evidence tending to show this to be true, though definite proof is still lacking. Anyhow, from the theoretical point of view the question is of considerable interest. As was demonstrated by AHLMARK (1944), as soon as

pregnancy has commenced the histaminolytic action of human plasma will rise from a very low, scarcely measurable rate to thousandfold. Ahlmark, as is known, has utilized this phenomenon in a quantitative test, by which it is rendered possible early to diagnose pregnancy and follow its further development with a view to prognosis.

Recently the question of a possible histaminolytic action of the skin has been actualized, viz. when NILZÉN (1947) in a study on the cutaneous histamine observed a considerable decrease in the histamine content of the skin on mechanical stimulation of normal skin and in certain cutaneous disorders arising on an allergic base. He suggested that this decrease might partly be due to a histaminase-histamine reaction. When surveying the literature available we found only a single paper dealing with histaminase in the skin (BEST and MCHENRY, 1930). These workers, however, had been unable to demonstrate a histaminolytic action of extracts prepared from dog skin. Hence, we thought it of interest to investigate the behaviour, in the above respect, of the skins of various animals and human subjects.

Our Own Experiments.

Procedure. — A skin specimen is weighed, divided into small pieces, and ground in a mortar with quartz sand and normal saline added. The homogeneous mixture is diluted with normal saline to 25 (50) ml, 25 (50) γ histamine dihydrochloride and 2—3 drops of toluene being added. This preparation is kept in a water bath at 37° C. for 24 hours. After centrifuging the histamine content of the precipitate is estimated directly with surviving isolated guinea-pig small intestine. In parallel experiments the mixture is boiled in the water bath for 10 minutes prior to being stored in the water bath at 37° C. for 24 hours. Further, in each experiment the histamine content of the skin is estimated with the BARSOUM-GADDUM technique (1935) as modified by CODE (1937).

The experiments were performed with material from guinea-pigs, rabbits, cats, and human subjects. As a rule, the skin specimens were taken from the abdomen.

It will be seen from Table I that guinea-pig skin exerts an appreciable histamine-destroying action.

As is shown by Table II, rabbit and human skin has an appreciable histaminase effect, which is however inconspicuous or absent in cat skin.

Table I.

Guinea-Pig No.	Weight of Specimen g	Histamine γ /g. skin	Weight of Specimen g	Histamine Added γ	Histamine Destroyed γ /g. skin
1	0.3060	8.0	0.6849	25	11.1
2	0.5445	4.5	1.0487	25	12.5
3	0.3045	8.9	0.7290	25	20.7
4	0.6121	9.6	0.7696	25	17.7 ¹
5	0.2000	10.6	0.2965	25	21.1 ²
			0.5757	25	16.1
			0.9255	25	13.5

Table II.

Rabbit No.	Weight of Specimen g	Histamine γ /g. skin	Weight of Specimen g	Histamine Added γ	Histamine Destroyed γ /g. skin
1	0.1900	10.5	0.6442	50	19.7
2	0.2370	14.7	0.5226	25	30.2
Cat No.					
1	0.3900	31.3	0.5688	50	0.5
2	0.3090	21.7	0.5990	25	5.8
Human Subject No.					
1	0.6498	8.5	0.9892	25	16.9
2	0.6074	7.7	0.6918	25	25.3

The control experiments in which the histaminase was destroyed by boiling the skin extracts after addition of histamine but prior to placing them in the water bath, yielded consistently negative results (*i. e.* no inactivation of histamine). In a couple of parallel experiments concurrent with the direct estimation with guinea-pig intestine, the specimens were extracted *in toto* according to Code. Also here conspicuous destruction was noted of the histamine.

In order to check the results of our investigation pertaining to the presence of histaminase in skin extracts, in some cases we have applied the same procedure to organs in which histaminase had already been demonstrated. In these experiments we used guinea-pig kidney, cat liver, and cat lung. The results are given in Table III.

¹ This rate was obtained after total extraction of the preparation according to Code.

² This and the two following rates relate to different specimens taken from one and the same animal.

Table III.

	Weight g	Histamine Content γ/g.	Weight g	Histamine Added γ	Histamine Destroyed γ/g. Tissue
Guinea-Pig Kidney	1.4116	6.8	1.5947	25	15.6
Cat Liver	2.0565	5.75	2.7345	50	27.2
Cat Lung	0.4500	40.0	1.0016	50	4.8

Table IV.

	Histamine γ/g. skin
Guinea-Pigs Nos. 7-13	3.03; 13.2; 20.9; 8.92; 8.58; 6.39; 15.6
Rabbits " 3-4	11.56; 9.45
Cats " 3-4	29.14; 39.7

In an additional series of animals the histamine content was estimated of the skin. The results tally well with the data given by TARRAS-WAHLBERG (1937) for the cat and rabbit, and by EMMELIN (1945) for the guinea-pig (Table IV).

Discussion.

From ACKERMAN's (1910) and subsequent, particularly AKER-BLOM's (1934), investigations the fact had emerged that certain bacteria are capable of converting histidine into histamine. In the first place, this action was noted in strains comprised in the *Salmonella* group. An inverted reaction, viz. histaminolytic action of certain bacteria, is also known (WERLE, 1941; BUCHERER and ENDERS, 1942; AHLMARK, 1944). In order to eliminate the disturbing influence of bacteria possibly present, toluene was used in the present investigation. This proved efficacious, as we were able to demonstrate by sterility tests in a number of instances, no growth being observed on agar and/or blood agar plates.

Provided a histaminase-histamine reaction does actually take place *in vivo*, the results presented above should be taken to justify the supposition that a reaction of this type is also possible in the skin. As was already pointed out, NILZÉN (1947) has demonstrated a decrease in cutaneous histamine in skin areas affected by urticaria. However, from the observations made by HORTON *et al.* (1932) it should be inferred that this decrease in histamine is not due exclusively to histaminolysis: when subjects with

urticaria provoked by cold are exposed to the latter, there is in addition to local and systemic symptoms a marked rise to be noted in the gastric production of hydrochloric acid. This experience indicates that the histamine liberated in the skin is not entirely inactivated but that a proportion enters the circulatory system. Likewise it might be pointed out in this connection that apparently the histamine reaction *in vivo* proceeds comparatively slowly, viz. as opposed to *e. g.* the inactivation of acetylcholine by the corresponding esterase.

Summary.

In the study recorded the present writers have demonstrated a histaminolytic action of extracts prepared from the skins of guinea-pigs, rabbits, cats, and human subjects.

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Studies on the Influence of Exercise on the Serum Iron in Man.

By

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During some work with haemoproteins and their clinical utilisation the author was interested in a statement by VANNOTTI (1939, 1942, 1946) that the serum iron content decreases after heavy muscular effort. VANNOTTI concludes that the muscular effort is followed by an enrichment of iron in the muscles from the circulating iron in the serum. If this observation were correct it would be of considerable interest for the study of the iron metabolism. It was therefore of importance to check the results.

In order to draw conclusions regarding the effect of heavy muscular work on the serum iron it is necessary to take into consideration the spontaneous changes in the serum iron level, which occur during the day (WAHLQUIST 1940). The effect of an effort of several hours duration may be masked behind the general decrease of the serum iron level during the whole day. This decrease has been found to amount to $36.3 \pm 9.2 \gamma \%$ (WAHLQUIST 1940). In the present investigation the acute effort lasted as regards the men for about one hour and as regards the women for about two hours. Serum iron determinations were made immediately before and after work. A new determination was made after another hour of complete rest. In order to increase the reliability of the determinations and also to get an idea of the error of the method

double determinations were carried out in all cases. There was also a study of the haemoglobin, red blood cell count and sedimentation rate in every case. The changes in reticulocytosis and haematocrite were followed as well as the serum iron. All determinations were carried out by the nurse in charge of the particular division of the Clinical Laboratory. Owing to the great extra burden thrown on the personnel by this investigation, a limit had to be put on the number of cases studied. It is obvious from the statistical treatment of the material that a far greater number of cases should have been studied in order to permit valid conclusions, as the standard deviation is rather big.

Method.

6 young healthy men (19—29 years old) and 7 healthy young women (19—33 years old) without signs or symptoms of disease were studied before and after severe exercise and after another hour of complete rest. The exercise was for the men, who all belonged to an athletic union (*«frisksportare»*); gymnastics, putting of heavy weights, running in Nylin's staircase at a rate of 50/208, bicycling against heavy resistance and so forth until a state of almost total exhaustion was reached. For the women a somewhat less strenuous exercise was chosen, viz. walking rapidly outdoors for about two hours.

A corresponding number of men (26—51 years old) and women (17—57 years old) were chosen from patients with either no disease or some circulatory disease in a well compensated state. Exactly the same serum iron and other determinations were performed on these patients at intervals, corresponding to those in the above-mentioned group, the patients being up and around but doing no exercise for one and two hours respectively and then resting for another hour. The studies were in most cases performed between noon and 18.00. No food was taken 3 hours before the tests or during the study.

The following tests were performed:

1. Before exercise:

Haemoglobin, duplicate, determined as acid haematin ($100\% = 15$ g Hb/100 ccs blood).

Red blood cell count.

Sedimentation rate.

Serum iron determination acc. AGNER (1947), duplicate.

Haematocrite, duplicate.

Reticulocyte count.

2. After exercise:

Serum iron determination, duplicate.

Haematocrite, duplicate.

Reticulocyte count.

3. After rest:
 Serum iron determination, duplicate.
 Haematocrite, duplicate.
 Reticulocyte count.

A description of the serum iron method is given by AGNER (1947).

Results.

73 duplicate determinations were performed and 4 single ones. The standard deviation (σ) of the differences between duplicate determinations was found to be $\pm 4.5 \gamma$. 3σ therefore is $\pm 13.5 \gamma$. The standard error of a single determination according to the formula $\pm \frac{\sigma d}{\sqrt{2}}$ was $\pm 3.2 \gamma$. If the total number of duplicate determinations was divided in three groups, viz. below 90 γ , between 90—180 γ and above 180 γ /ccs serum, the standard error of single determinations was correspondingly $\pm 1.1 \gamma$, $\pm 5.3 \gamma$ and $\pm 2.4 \gamma$. Provided 5 determinations outside $\pm 3 \sigma$, which are regarded as unreliable, were excluded, the figure $\pm 5.3 \gamma$ is reduced to $\pm 2.6 \gamma$ and the standard error of a single determination on the total material to $\pm 1.9 \gamma$.

The results with regard to the differences in serum iron content (mean values from duplicates) before and after work and after rest in the different groups is seen in table I. As there was no significant difference between the determinations in men and women it has been considered justified to compare the complete groups of men and women doing exercise with the groups doing no exercise. There was no significant change in the haematocrites

Table I.

Difference in serum iron content before and after exercise and after rest, expressed as % of the value before exercise.

	1)	2)	3)
	After exercise Before exercise	After rest After exercise	After rest Before exercise
Groups, doing exercise (6 males, 7 females in 1), 6 males, 6 females in 2) och 3).			
Difference (%)	-1.1 ± 2.9	$+3.4 \pm 3.5$	$+3.3 \pm 4.8$
St. deviation	± 10.5	± 12.2	± 16.6
Groups, not doing exercise (6 males, 7 females)			
Difference (%)	-4.8 ± 2.5	-3.2 ± 1.9	-7.8 ± 3.0
St. deviation	± 9.0	± 6.8	± 10.8

in either group during the whole experiment, nor was there any correlation between the direction of change in the haematocrite and in the serum iron. As the serum iron is by definition calculated as γ iron per 100 ccs. serum it is not necessary to make individual corrections for haematocrite values. If, however, the exercise is accompanied by a selective loss of iron-free or iron-poor fluid from the blood to the tissues, affecting the blood composition so as to increase the haematocrite (NYLIN 1947) by some percent this may appear as a relative increase in serum iron content. According to NYLIN's (1947) studies there is little reason to believe in an increased output of red cells during exercise.

In spite of the severe effort, especially in the male group, the increase in reticulocytosis was moderate, and in the women even absent. Details are seen in table II.

Table II.

Average number of reticulocytes ($\%$) before and after exercise and after rest.
(No statistical analysis made on account of the small numbers.)

	Before exercise	After exercise	After rest
Men doing exercise (6 cases)	3.4	4.7	5.7
Men, not doing exercise (6 cases) .	5.0	5.2	5.8
Women, doing exercise (7 cases) ..	5.6	5.6	5.1
Women, not doing exercise (7 cases)	4.3	5.4	4.4
Groups doing exercise.....	4.6	5.2	5.4
Groups, not doing exercise	4.6	5.3	5.1

Comments.

The values of the differences in serum iron content in table I are given as $\%$ change of serum content. The initial values for serum iron varied widely (from 49 γ to 279 γ per 100 ccs. serum). Calculation of differences in absolute values have been made, but show a greater standard deviation than the figures presented in table I. The direction of the change was mainly the same. It is seen, that no difference in the whole material is significant. The actual figures however show, that in the non-exercising group there is a gradual decrease in serum iron content between the three determinations. Although this is true to a much lesser extent for the exercising group during exercise, there is an increase of the serum iron content during the hour of rest to such an extent that at the end of the experiment there is a total increase of serum iron content.

Conclusions.

Severe exercise for one—two hours did not affect significantly the serum iron content. The limited material at hand (26 cases) showed so great variations even under standard conditions that the results cannot be regarded as statistically significant. There was, however, no proof that exercise brings about a decrease of serum iron content; the actual figures point to the opposite.

Summary.

An attempt was made by duplicate determinations of serum iron in 13 cases (6 males, 7 females), who did severe exercise, and another 13 controls (6 males, 7 females), who did no exercise, to investigate the fate of serum iron during and after exercise. Owing to great individual variations in both directions and a correspondingly great standard deviation no significant changes were observed, neither in the groups, nor between them.

Acknowledgements.

The author owes sincere thanks to the nurses Maj Lundström, Britta Sandin and Märta Wallin for their skilful work and readiness to cooperate in this study.

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Interaction of Ergotamine and Carbon Dioxide on Blood Pressure and Respiration.

By

ÅKE LILJESTRAND.

Received 23 December 1947.

In the narcotized cat large doses of ergotamine give adrenaline reversal or reversed effect of stimulating the splanchnic. But smaller doses which do not diminish the response to adrenaline, nevertheless interfere greatly with the regulation of the blood pressure. Thus HEYMANS and REGNIERS (1929) and WRIGHT (1930) found that 0.1—0.25 mg per kilogram bodyweight abolished vasomotor reflexes from the carotid sinus in the dog and cat. On the other hand WRIGHT showed that 0.1—0.15 mg per kilo in the cat was insufficient to depress the effect of injected adrenaline or otherwise block the peripheral vasomotor mechanism. After it had been established that the pressor effect from the sinus region also could be elicited by chemical stimuli, EULER and SCHMITERLÖW (1944), made a closer study of the effect of ergotamine at this point of action. They found that small doses selectively abolished the pressor effect elicited by decreased pressure in the sinus, *i. e.* arising principally from the baroreceptors, whereas the effects on blood pressure and respiration produced by such chemical stimuli as hypoxia, cyanides or nicotine remained.

While asphyxia or the administration of carbon dioxide rich mixtures to the narcotized cat give only insignificant changes of blood pressure, GANTER (1926) showed that the result of these procedures after small doses of ergotamine was a distinct drop of blood pressure. Recently GERNANDT and ZOTTERMAN (1946)

recorded the efferent impulses in the splanchnic nerve in cats which had been treated with 0.05—0.1 mg ergotamine per kilogram. Although the blood pressure reaction to the inhalation of carbon dioxide rich mixtures or to asphyxia was changed after ergotamine injection into a fall of blood pressure, they found that the splanchnic efferent outflow was not influenced by the ergotamine, an increase of impulses being obtained in both cases quite as before the injection. The drop of blood pressure accompanying asphyxia was shown principally to be due to the carbon dioxide accumulation. Discussing their results GERNANDT and ZOTTERMAN concluded that the increased splanchnic efferent outflow after ergotamine and carbon dioxide probably contributed to the vasodilatation through liberation of sympathin I. A further study of this problem seemed indicated.

Methods.

Cats were narcotized with 0.05—0.06 g chloralose per kilogram bodyweight. The blood pressure was recorded with mercury manometer usually from the femoral artery but in some cases from the carotid artery. When the blood pressure alone was recorded artificial respiration with a Starling pump was generally used. Carbon dioxide was given in oxygen or in air from bags. No important difference in the blood pressure reaction was seen between natural or artificial respiration. When the major splanchnics were prepared this was done from the back of the abdomen. In these cases usually one of them was severed before beginning the experiment. For the investigation of effects on the respiration quantitative measurements were made with the body pletysmograph described by EULER and LILJESTRAND (1936). The gas mixtures were then administered through the inspiratory Müller valve. Nerve stimulation was carried out with faradic current. The ergotamine used was the preparation Gynergen Sandoz (ergotamine tartrate) and it was always given intravenously.

Results.

The administration of 7 or 15 per cent carbon dioxide usually only gave insignificant changes of blood pressure. After 0.01—0.2 mg ergotamine tartrate per kilo the stronger carbon dioxide concentration caused a pronounced fall of blood pressure. If a fall had already occurred before the ergotamine administration, the drug increased this fall. The smaller carbon dioxide concentration usually gave a drop of blood pressure though less pronounced. The fall after carbon dioxide administration was

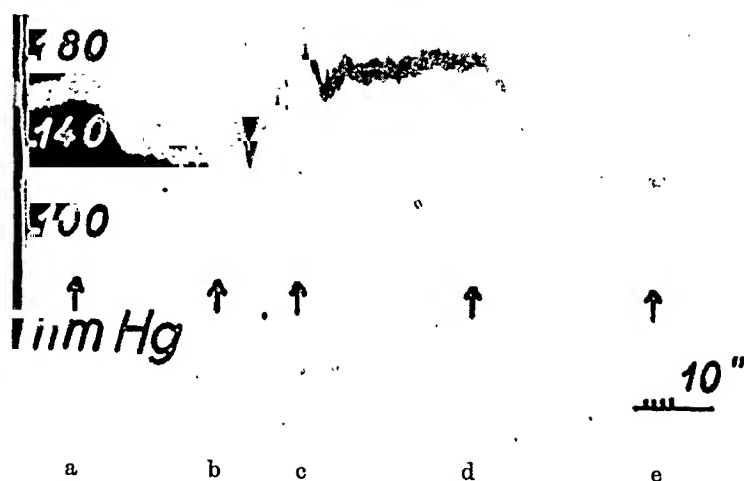


Fig. 1. Cat 4 kg. Left splanchnic cut. Artificial respiration. 0.4 mg ergotamine tartrate. Between a and b inhalation of 7 % CO_2 in O_2 . The blood pressure falls from 164 to 132 mm Hg. At c the right splanchnic is severed. This is followed at d by inhalation again of 7 % CO_2 . The blood pressure now falls from 180 to 124 mm.

sometimes equally large when the gas mixture was given during the phase of greatly increased blood pressure in the minutes following the ergotamine injection as when as usual given after the blood pressure again had returned to a more normal level. After section of the splanchnics the blood pressure fall from carbon dioxide was either unchanged or increased. This is illustrated by fig. 1. Evidently the increase of the splanchnic efferent outflow can play no deciding rôle in the origin of this blood pressure drop.

The fact that the fall of blood pressure after administration of carbon dioxide to ergotaminized cats is quite as apparent after severing the splanchnics as before, whilst this carbon dioxide inhalation still gives a distinct increase in the splanchnic efferent outflow in spite of the ergotamine, affords strong reasons for believing in the existence of a peripheral blockade. However, the importance of the splanchnics for the regulation should not be overestimated. If, after bilateral splanchnicotomy, the animal is given the carbon dioxide mixture or if it is asphyxiated no fall of blood pressure or only a moderate fall is obtained, whereas it becomes pronounced after ergotamine. This is seen in fig. 2. Evidently the blood pressure may be regulated quite well through other nerves.

As the drop of blood pressure could not be considered to be due to the augmented splanchnic activity, it seemed probable that

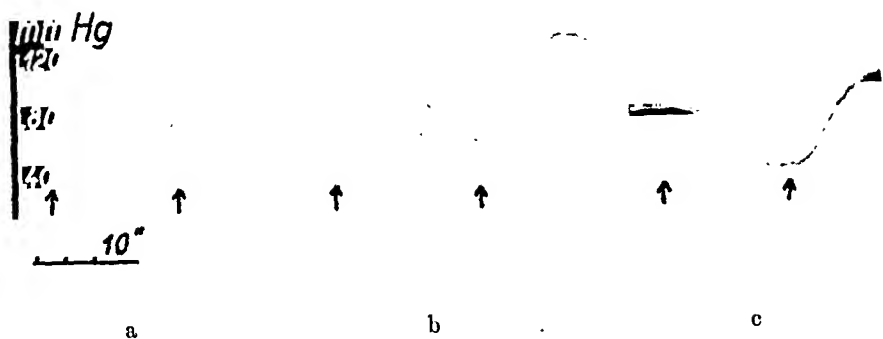


Fig. 2. Cat 2.4 kg. Thorax opened. Artificial respiration. Both splanchnics severed. Between the arrows the respiration is stopped (45 sec.). a) Blood pressure 74 mm Hg. Between a and b 0.24 mg ergotamine tartrate is given. During the increased pressure that ensues, asphyxia gives a fall of blood pressure down to 60 mm Hg (b). c) Nine minutes later the blood pressure falls from 112 to 54 mm Hg.

it was caused directly by the carbon dioxide, while the usual mechanism of compensation was blocked, centrally, peripherally or both. Certainly a nervous vasodilatation may be produced on other ways than through the splanchnics. In the present case such a vasodilatation becomes less probable as other blood pressure reflexes were still normal. Thus, a rise of blood pressure practically as large as before ergotamine administration is still elicited from the chemoreceptors of the sinus region by potassium cyanide for example (EULER and SCHMITERLÖW). A peripheral block ought to give increased effects for other vasodilating substances too. In the present experiments, however, injection of single doses of acetylcholine or histamine gave no consistently larger effect after ergotamine than before. In no case was the amplitude augmented. In some cases, on the contrary, it decreased. Often, although not always, the blood pressure fall was somewhat prolonged, however. Fig. 3 d and e show results from one of the experiments illustrating these effects.

With these rapid falls of pressure there is the possibility that even normally the principal compensation is not performed by nervous regulation because of the inertia of the regulating mechanisms, but by a local mechanism through return to a mean tonus position of the vessels after the disappearance of the vasodilating substances. With continuous infusion on the other hand a nervous compensation might be expected to play a part. The effect of continuously administered histamine or acetylcholine, however,

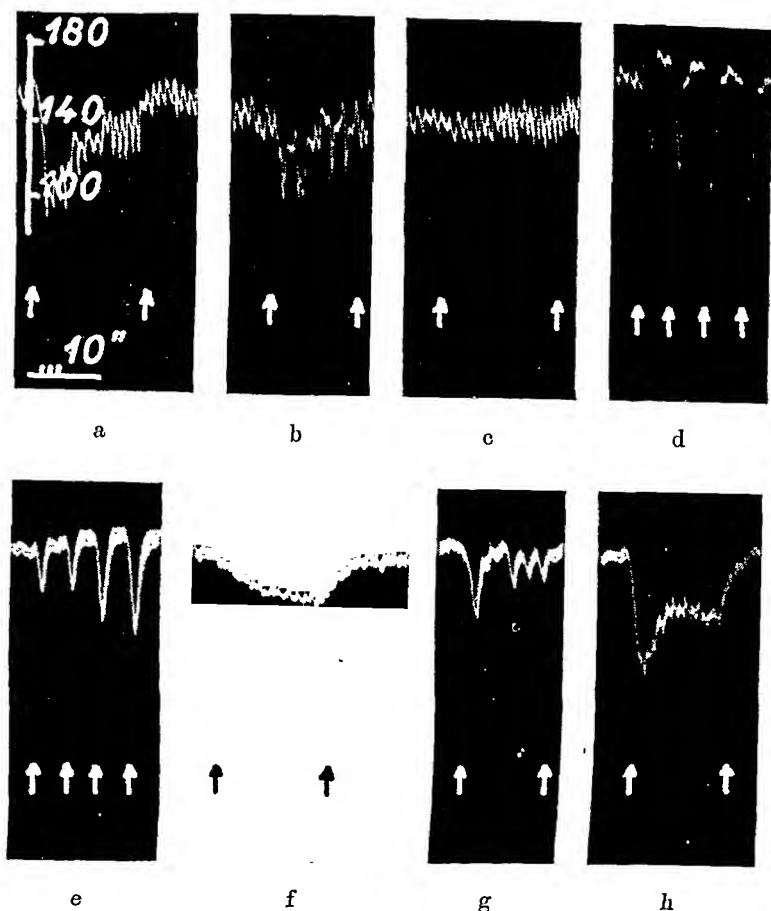


Fig. 3. Cat 3.5 kg. Artificial respiration. Between d and e 0.35 mg ergotamine tartrate is given.

a) and h) continuous infusion during two minutes with 12.5 γ histamine per minute.

b) and g) continuous infusion during two minutes with 2.5 γ histamine per minute.

c) and f) inhalation of 7.3 % CO_2 .

d) and e) injection of 1, 2, 4 and 8 γ histamine respectively.

was unchanged or only slightly increased after ergotamine, the results being quite insufficient to explain the drop of blood pressure after carbon dioxide. This may be seen in fig. 3 a, b, g and h. The ergotamine also might influence the vasoconstrictors locally so that their effect is decreased at raised levels of carbon dioxide although it remains normal in other ways. But the effect of splanchnic stimulation was not changed during inhalation of carbon dioxide mixtures in ergotaminized cats. On the other hand in one experiment the effect of splanchnic stimulation during air

inhalation was diminished after 0.1 mg ergotamine per kilo. (See fig. 4.) As a rule other investigators have found no decreased effects after such small doses of ergotamine. But the rather low blood pressure in this case may indicate that a certain degree of shock has occurred, and the result then becomes more understandable, the animals becoming more sensitive to ergotamine during shock as shown by SMITH (1928) in the dog.

As the results up to this point mostly indicated an essentially central inhibition of the blood pressure response to carbon dioxide, attention was directed to the effect of ergotamine on the respiratory response to carbon dioxide. It was found in several experiments that 0.01—0.1 mg ergotamine per kilo clearly dimin-

ished the ability of the animal to increase the respiration on administration of carbon dioxide mixture. As a rule the effect was discernible with 15 per cent carbon dioxide and it sometimes appeared with 7 per cent. The changed response was principally noticeable in the depth of respiration while the frequency was hardly changed. During the minutes following ergotamine injection the spontaneous respiration of air was also decreased and in some animals this decrease persisted for at least one hour. The diminished sensitivity to carbon dioxide remained present for some hours the effects on the respiration thus persisting much longer than the corresponding influence on the blood pressure. At the same time a displacement of the mean position to the inspiratory side was seen during inhalation of the carbon dioxide mixtures. Table 1 gives some values from one experiment. EULER and SCHMITERLÖW have shown that doses of ergotamine of the order concerned here do not interfere with the regulation of respiration in response to oxygen lack, cyanides or nicotine. Their results with oxygen lack or potassium cyanide were confirmed in the present experiments. Evidently a specific, central inhibition of response to carbon dioxide must exist.

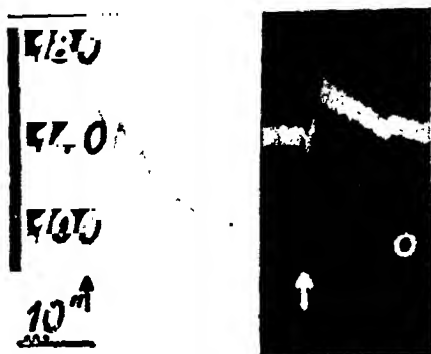


Fig. 4. Cat 3.8 kg. Stimulation of the right splanchnic. Between a and b 0.38 ergotamine tartrate is given.

Table 1.

Cat 3.7 kg. Test of 14.9 % CO₂ in O₂.

Time	Blood pressure in mm Hg			Ventilation in ml per min.	
	Air-respiration	CO ₂ -respiration	Change	Air-respiration	CO ₂ -respiration
10.41—10.44	142	142	0	816	2,700
10.48	0.05 mg/kg ergotamine				
10.54—10.57	166	114	— 52	425	2,290
11.27—11.30	140	116	— 24	680	1,490
11.47—11.50	130	118	— 12	698	1,470
12.10—12.13	146	136	— 10	602	1,580
13.12—13.15	122	138	+ 16	836	1,680
14.13—14.16	118	138	+ 20	840	2,080
14.58—15.01	120	134	+ 14	990	2,120

Discussion.

From this and earlier investigations it appears that small doses of ergotamine have pronounced effects on the regulation of the respiration and blood pressure in response to gas mixtures rich in carbon dioxide. All facts speak for the origin of the changes in the respiration in the respiratory centre, as normal sensitivity of the chemoreceptors in the sinus region can be shown for potassium cyanide and for oxygen lack. Investigations of EULER (1938) who found decreased sensitivity of the respiratory centre to potassium ions after ergotamine administration, are in agreement with this view.

The change of blood pressure regulation in response to carbon dioxide respiration seems less clear. The interpretation becomes difficult on account of the large individual differences in sensitivity and because the number of experimental manipulations on any one animal is necessarily restricted. The increased splanchnic activity found by GERNANDT and ZOTTERMAN speaks in favour of a peripheral blockade but this influences the blood pressure only very slightly or not at all. A central inhibition specific for carbon dioxide is made probable by the fact that the effect to other vasodilator substances is not greatly increased; by the fact that another central inhibition for carbon dioxide has been shown *i. e.* on the respiration; and lastly by the fact that normal blood

pressure reactivity of the sinus chemoreceptors is present with these doses of ergotamine (EULER and SCHMITERLÖW). Further, EULER and LILJESTRAND (1946) have shown that a larger drop of blood pressure is obtained after ergotamine, if the sinus and aorta regions have been previously denervated.

Strong reasons therefore seem to exist for the view that the fall of blood pressure after administration of carbon dioxide to the ergotaminized cat is caused by local vasodilating action of carbon dioxide, its influence being uncompensated principally because of an inhibition of the vasomotor centre. The peripheral blockade of the vasoconstrictors is less important. Carbon dioxide is already known to produce an appreciable fall of blood pressure if the normal regulation is eliminated, as was shown by BACQ, BREMER, BROUHA and HEYMANS (1939), on the totally sympathectomised cat.

Summary.

The action of ergotamine on the effect of inhaled carbon dioxide on blood pressure and respiration in the cat has been investigated.

The results indicate that the fall of blood pressure caused by carbon dioxide in the ergotaminized cat is principally due to a central inhibition of the blood pressure regulation, the inhibition being relatively specific to carbon dioxide. The local vasodilating action of the carbon dioxide then appears.

Ergotamine was also found to exert an inhibitory influence on respiratory regulation. It caused a decrease both in the normal ventilation and in the extent of the usual increased ventilation following inhalation of carbon dioxide. This action is interpreted as arising from a diminished sensitivity of the respiratory centre.

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Studies in the Electric Excitability of Peripheral Motor Neurons and in the Factors Which Constitute the Electric Excitation in these Neurons.

By

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Introduction.

The experiments which will be described in this paper may be regarded as complementary to a work previously carried out by one of the authors (G. F. G.) and reported both in Swedish (1907) and in a German version (1909). This previous investigation showed by means of experiments carried out with accurate measuring apparatus upon frog nerve muscle preparations (sciatic-gastrocnemius) that a shock applied unipolarly to the nerve from a contact of a potentiometer and conducted through the nerve and its associated muscle to one plate of a condenser of known capacity, induces in the muscle a barely perceptible twitch at a much lower voltage if the shock has negative than if it has positive potential. The experiments showed also that a shock of *negative* potential which is conveyed from the potentiometer through the nerve muscle preparation to the condenser, induces a barely perceptible twitch of the muscle at a much lower potential if the direction of the stimulating shock in the nerve is musculopetal than if it is musculofugal.

The procedure employed in arriving at the latter result must be described again in this paper in order to show how far the previous

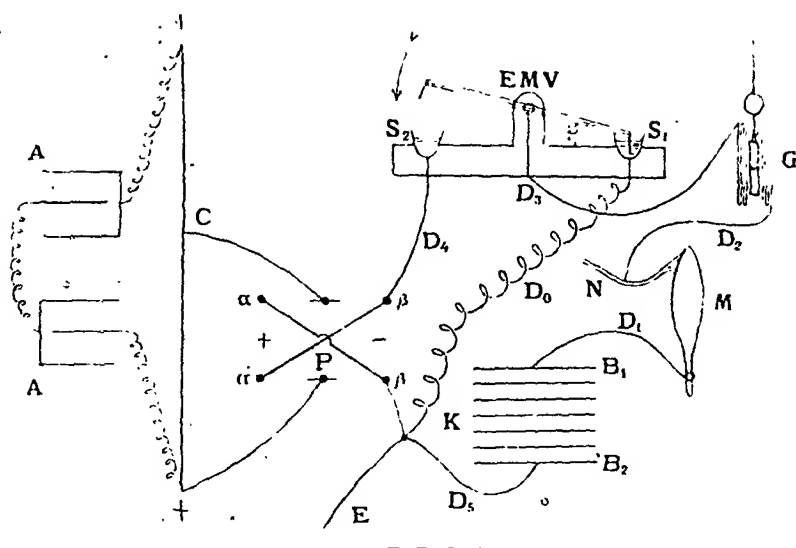


Fig. 1. The experimental device used by GÖTHLIN (1907, 1909) for determination of the muscle threshold on exciting a sciatic-gastrocnemius frog preparation by single shocks of different signs and different directions in the nerve.

results are comparable with those obtained in the experiments which we are now reporting; we therefore reproduce a sketch of the apparatus and a description. (See Fig. 1.)

Two accumulators (AA) generate a p. d. of 4000 mv. in a platinum wire extending over a 1000 mm scale between the signs + and — on the graph. The charging potential is chosen by means of a sliding contact (C), and this potential may be made negative or positive by means of a Pohl reversing switch which alters the connection to ground. K is a condenser, G a ballistic galvanometer (only employed in control experiments); S_1 and S_2 are glass cups containing mercury. S_1 is connected to ground by D_0 and S_2 by D_4 to the impulse generator. In the reversing apparatus EMV a bar of ebonite carries a platinum wire ending in platinum contacts for the mercury, while the wire D_3 conducts the impulse — negative or positive — from the middle of the platinum wire to the nerve-muscle preparation (NM) enclosed in a moist chamber.

When contact is made at S_2 , a measured quantity of electricity traverses the nerve towards the muscle and is collected by the condenser B_1 . When the current was to pass from muscle to nerve, the conducting wires D_1 and D_3 were changed over. From the diagram it is evident that the preparation is in connection with the ground until a moment before the time when the shock reaches it.

Experiment 7. ²⁰/7, 1906. *Rana esculenta*; wt. 38 gm. The preparation is placed in the moist chamber 5.25. A stream of air saturated with

Table I.

Sign of electricity; direction of its propa- gation in the nerve between the electrode and muscle	Potentiometer potential at the threshold, in millivolt	Voltage- capacity thresh- old in 10^{-9} coulomb	Time of observation
+ E ↓	700	0.7	5.40
- E ↓	296	0.296	5.44
+ E ↓	692	0.692	5.48
- E ↓	296	0.296	5.50
+ E ↑	356	0.356	5.54
- E ↑	676	0.676	6.00
+ E ↓	692	0.692	6.05
- E ↓	324	0.324	6.09

↓ = condenser charge passes the nerve in *musculopetal* direction
 ↑ = condenser charge passes the nerve in *musculofugal* direction

water is passed through. Temp. 20.1° — 20.0° . 1.5 cm. of the nerve lies below the electrode and 2.2 cm. above it. The capacity of the condenser is 0.001 mf. Threshold voltage determined with an accuracy of 4 mv.

The figures in the report on experiment 7, and in all the experiments in this series, show that a shock of negative potential directed in the nerve towards the muscle reaches the twitch threshold when its potential is only about half that required to achieve the same effect when the direction of the shock is away from the muscle.

The new series of experiments which will be reported in this paper were designed to elucidate whether the greater excitatory effect of a stimulation by a single shock of negative potential in the case of musculopetal, as opposed to musculofugal shock, is also manifest if physiological conditions are simulated by using a series of stimuli applied at regular intervals appropriate to the nerve.

Method.

The projected investigation required first and foremost a new apparatus which would provide shocks of a suitable form and at a suitable frequency. Our first essential requirement was that the configuration of each electric impulse should be of the type (see Fig. 2) obtained when one isolates that $1/4$ of a sinusoidal wave, in

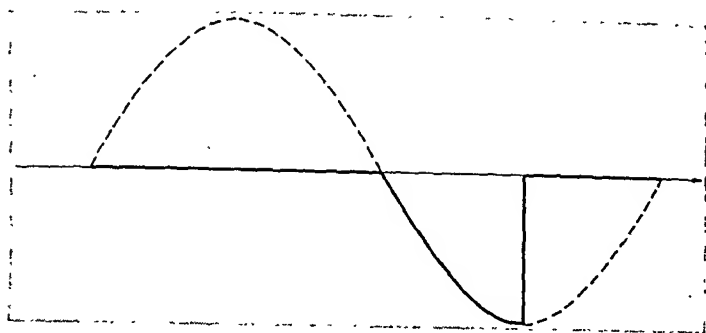


Fig. 2. Diagram to explain the idea of abolishing part of a sinusoidal wave and leaving behind only that $1/4$ period which is characterized by increasing voltage of negative sign.

which the potential begins to become negative and then grows from 0 to its peak; the wave is then cut off so that the apparatus is at zero potential during the remaining $3/4$ of the wave.

We also required that the duration of the $1/4$ wave should be sufficient for the *temps utile* of the preparation, and that the maximum potential should be not less than 0.4 volts. Finally, we required that the apparatus should deliver 50, 75, 100, 200 and 300 pulses per second.

The construction of the apparatus was undertaken by Svenska Radioaktiebolaget, Stockholm. Engineer Inge Svedberg devised the principle of the construction, the main features of which he describes as follows:

The impulse generator is a pulse oscillator giving a voltage corresponding to the proposed quarter of a cycle of a sinusoidal voltage and works as follows. A sinusoidal oscillator *O* feeds a circuit containing a diode *D*, a resistance R_1 , and a potential divider R_2 in series. Parallel to this circuit is a neon tube *N*.

Starting at a moment when the voltage at the point *a* (v. Fig. 3) is positive relative to *b* (or *G*, which is ground), we see that no current flows through the diode and accordingly no voltage is developed across the out-put terminals *V—G* of the potentiometer R_2 . When the voltage of *a* becomes negative relative to *b* the current flows through R_2 and a sinusoidal voltage is built up across *V—G*. When the voltage *a—b* has grown to the striking voltage of the neon tube *N*, this tube suddenly becomes conducting and short-circuits the oscillator *O* so that the voltage across R_2 immediately drops to zero.

In the apparatus used for the tests the neon tube is a gasfilled triode (thyatron) and the striking voltage can be regulated by varying the d. c. grid-bias. If the bias is so adjusted — an adjustment which is carried out under the control of a milliammeter *mA* — that the current barely begins to flow through the neon tube at the moment when the maximum value of the cycle is reached, the rest of the cycle is cut away, leaving only the desired $1/4$ of the cycle at the out-put terminals.

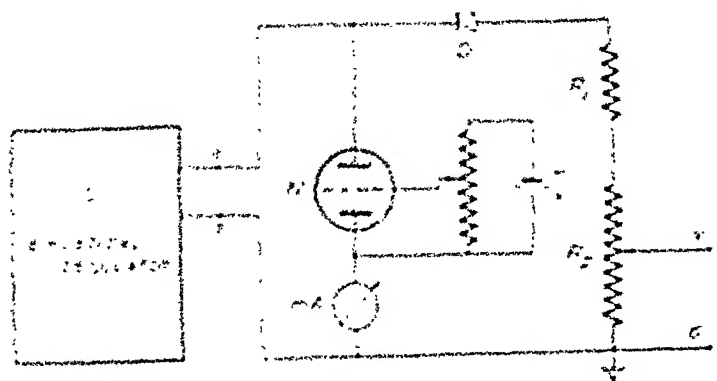


Fig. 2. Lay out of the impulse generator.

It may be noted that the neon tube is not an absolute short-circuit but that it gives a voltage drop of about 15 volts when conducting. This means that the voltage across the diode and associated resistances R_1 and R_2 will not drop immediately to zero but to 15 volts when the tube strikes. In the actual apparatus this voltage is compensated for by giving the diode a negative bias of a corresponding value.

The oscillator is a vacuum tube working with an ordinary LC circuit, the feedback being obtained with an auxiliary tube.

The necessary supply voltages for anode circuits, heaters and grid bias are obtained from the A.C. source through rectifiers and stabilizing resistors.

The sinusoidal output of the oscillator is obtained in three ranges: 10, 100, 1000 cycles/sec. and 1000-5000 cycles/sec. within which the frequency is varied in steps of 10, 100 and 1000 respectively. The sinusoidal output from the oscillator may also be connected directly to the output terminals (i.e. the LC parallel-forming circuit may be disconnected).

A few significant data regarding the functioning of the impulse generator may be added to the description by the authors. They have recorded the wave form of a single shock emitted by the generator by means of a vacuum tube cathode ray oscillograph and very fast photographic amplification (T. A. M. 1947). The oscillogram is reproduced in Fig. 3.

The oscillogram in Fig. 3 shows that the effective portion of the wave, which is above the negative potential, occupies the full period

pos.

neg.

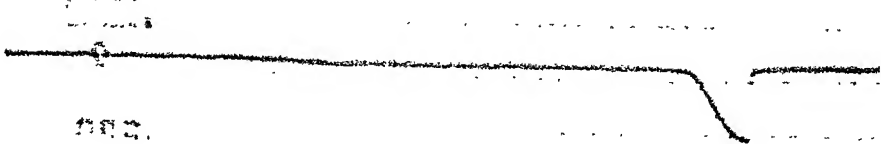


Fig. 3. Oscillogram of a single impulse as obtained by the impulse generator described by the authors. The negative portion of the wave is shown above the horizontal line.

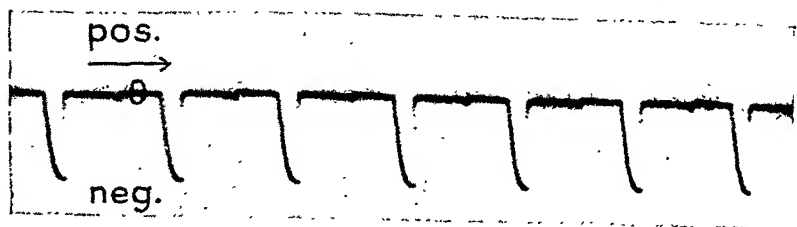


Fig. 5. Photograph of a series of impulses from the impulse generator at a frequency of 200/sec., registered by a v. Ardenne oscillograph. Speed 2.72 m/sec.

while the time taken for the return to the base line is not measurable and is certainly less than 0.1 millisecond. Another oscillogram was made with slightly slower paper while the apparatus was emitting a series of impulses at a speed of 200 per sec.

The potential generated can by means of the finely adjustable potentiometer be raised sufficiently gradually for our purpose from 0 to 0.4 volt, which is its maximum output.

A surviving motor neuron preparation of frog (GÖTHLIN).

A frog is decapitated by a transverse cut across the skull between the corners of the mouth. In order to remove the remnants of the medulla oblongata from the spinal cord, two symmetrical scissor incisions are made under the skin on each side from a point on the decapitation line midway between the midline and what was the corner of the mouth. These incisions are taken as far as a point on the vertebral column at a level with the posterior edge of the ear drums, thus freeing a triangular piece of bone which contains the remains of the medulla oblongata. Bleeding can be ignored. The operation leaves a preparation of a spinal animal which should display no "posture correction reflex".

In extending the decapitation incision, the skin is cut with scissors as far as the ventral midline, and is then incised along the ventral and dorsal midlines so that the incisions meet at the symphysis. The skin is peeled off on each side as far down as the symphysis, after which it is peeled off each leg down to the toes while the body is held in place by a strong pin against the symphysis.

The skinless preparation is placed on its back and the abdominal and thoracic wall cut through in the anterior midline. The viscera are removed with care to avoid damage to the sciatic plexus. The plexus on one side is then carefully isolated from the vertebral column to the thigh.

The next stage requires a glass rod one end of which tapers gradually and terminates in a knob. While the bone of the lower leg on the side selected is held fast with forceps, the glass rod is introduced between the gastrocnemius and peroneal muscles and separates them from each other along their entire length. At the upper and lower ends of the space thus made, the bone is cut away with its attached muscles so that the foot is only held to the thigh by the gastrocnemius. The muscles of the

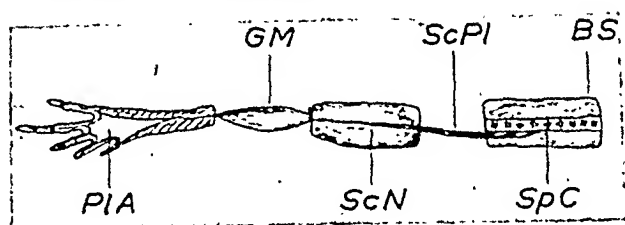


Fig. 6. Diagram of a frog motor neuron preparation. SpC the intact spinal cord in the unopened vertebral column; ScPl the sciatic plexus; ScN the sciatic nerve, embedded in the thigh musculature; GM the gastrocnemius muscle; PIA plantar aponeurosis.

lower leg and foot which have not been removed in cutting away the bone can no longer be induced to contract by stimulation of the sciatic plexus or nerve.

The pelvis is cut through at the symphysis. While the sciatic plexus on the chosen side is carefully raised with a glass hook, the posterior abdominal wall is cut through just below the point where the sciatic plexus emerges and immediately above the point where the plexus becomes the sciatic nerve and the latter enters the musculature of the thigh. Finally, two incisions are made with scissors from the abdominal region up to the neck parallel with and barely 1 cm from the vertebral column. Everything not attached to the column is then removed. If, as in this case, the object of the preparation is to display the smallest possible perceptible contraction of gastrocnemius, it is a disadvantage that the thigh muscles should be connected by nerves with the sciatic plexus and be able to twitch when the plexus is stimulated. This is prevented by careful exploration between the muscles of the back of the thigh and cutting the motor branches of the sciatic nerve which leave its upper part. The nerve is then replaced in its tunnel between the muscles.

A preparation made on these lines contains all the motor neurons with motor end-plates in the gastrocnemius which arise from anterior horn cells on the side concerned. If one electrode is placed high on the paravertebral muscles of the preparation and the other on the plantar aponeurosis of the foot, all these neurons throughout their length will lie within the course of a current that flows between the two electrodes.

It should be observed that when electrodes are placed in this way on a preparation of this kind, the cross-sectional area of the osseous and muscular parts surrounding the spinal cord is at least 30 times that of the sciatic plexus, and the cross-sectional area of the gastrocnemius at least 40 times that of the plexus. The current density, variations in which determine the stimulation of the tissues, will be correspondingly greater in the sciatic plexus than in the anterior horn cells or the end-organs of the neurons in the muscle. Consequently, when the threshold is sought by single shocks through electrodes placed on it in the manner described, the response of the gastrocnemius (if it responds) must be due to stimulation of the sciatic plexus and not of

the anterior horn cells or of the branches of the sciatic nerve in the muscle.

For some purposes a symmetrical double preparation may be required, in which case both hind legs are prepared in the manner described. Both sciatic plexuses are retained, the whole of the posterior abdominal wall below their exit is removed and each leg of the preparation remains attached to the vertebral column by its sciatic plexus only.

For the experiments to be described in this paper, the preparation is placed carefully on a suitably sized slab of paraffin wax of low melting point. The preparation is placed on its back but the lower leg and foot are rotated through 90° so that the latter's plantar surface faces upwards. Care is taken to ensure that the sciatic plexus, without being unnecessarily stretched runs a straight course across the slab.

The preparation is fixed in this position by means of a hedgehog quill passed through the thigh — but carefully avoiding the sciatic nerve — and another through the sole of the foot.

Choice of frequency.

The frequency of the shocks which are employed to stimulate the neuron preparation has been 75/sec. throughout. The choice of this frequency is based upon earlier results of special investigations into the action waves of frog muscle. F. BUCHANAN (1901) recorded their frequency in the sartorius muscle of frogs with a capillary electrometer during reflex tetanus in animals treated with strychnine. These observations led to the following result, among others (p. 149): "The average frequency as estimated from such curves as were measured — the duration in 37 periods in the muscle-responses of 9 frogs were accurately measured — was one of about 68 per second." The choice of the frequency 75/sec. is also based upon determinations of the inherent rhythm of frog muscle carried out by DITTLER and TICHOMIROV (1908) who found it to be about 60 per second in winter frogs and about 100 per second in summer frogs.

In separate experiments, the authors found that the flow of a series of impulses from the generator through the sciatic plexus of a neuron preparation at a frequency of 75 per second induced a very even tetanus in the gastrocnemius muscle.

The experimental circuit as a whole.

Fig. 7 gives a general idea of the circuit with which the neuron preparation was investigated. The following is an explanation of the device using the symbols attached to the various components of the circuit.

Between the terminals of the impulse generator is applied a du Bois-Reymond short-circuiting switch which is only opened when current is to be fed to the preparation. C is a finely adjustable com-

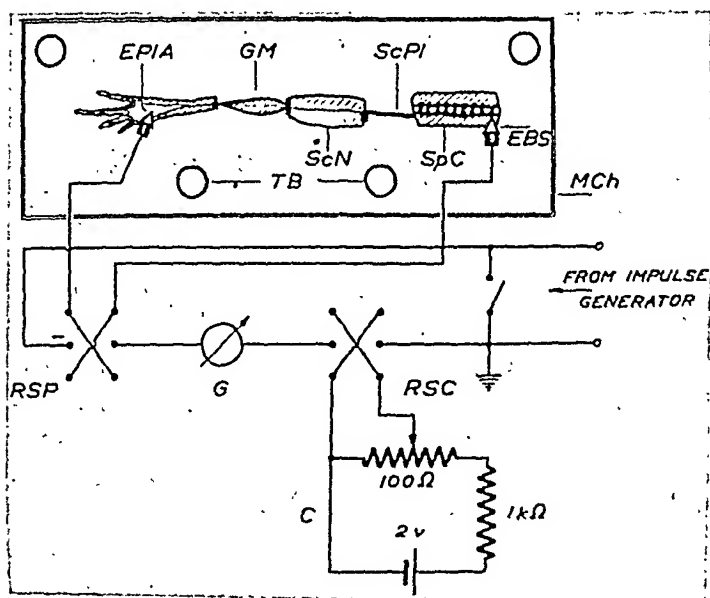


Fig. 7. Diagram of experimental arrangement for determining the muscle threshold on exciting motor neurons by periodic shocks of negative potential travelling in different directions in the nerve. Explanation of the letters is given in the text and partly in connection with Fig. 6.

pensator, and RSC its reversing switch. G is a multiflex galvanometer of the type devised by B. Lange; a direct reading scale, internally illuminated, and a high degree of damping permit rapid reading. In the instrument used by us, one unit on the scale (*i. e.* 1 mm.) corresponded to a current of 7.5×10^{-9} amps. Its resistance was 975 ohms. RSP — like RSC — is a "double pole double trough switch" (LEEDS and NORTHRUP) which reverses the current to the preparation.

MCh is a moist chamber with plain glass walls which were coated with a very thin layer of soft soap before every experiment in order to permit better vision. The dimensions of the chamber are: length 60 cm., width 32 cm., height 30 cm. TB are four small Dewar bottles filled with water very nearly at boiling point. The neuron preparation lies on a slab of paraffin wax and is held in position on it by hedgehog quills. At EBS and EPIA the current is conducted to the preparation by non-polarising electrodes of the du Bois-Reymond type ("Tonstiefelektrode"). EBS is placed upon the undamaged surface of the paravertebral muscles and EPIA upon the plantar aponeurosis.

The e. m. f. of the preparation, including the electrodes, is measured and compensated immediately before every determination of threshold, the short circuit switch being closed during this measurement and the galvanometer adjusted to zero with the help of the compensator. The threshold of the preparation is then determined at once by opening the short circuiting switch — with the impulse generator's potentiometer adjusted to zero — and then very carefully increasing the

potentiometer setting until an assistant notes the first perceptible contraction of the gastrocnemius. This is repeated twice and the galvanometer is read on the second occasion.

Experiments and Numerical Results.

All the experiments were carried out on specimens of *Rana temporaria* caught during the summer.

Experiment on the 14.11.45. *Rana temporaria*; wt. 23 gm.; *bilateral* spinal preparation.

Preparation of the animal began at 13.15 hrs and the neuron preparation was inserted in the moist chamber at 13.40 hrs. The electrodes were placed on the plantar aponeuroses of the two feet. The term right—left signifies that shocks of negative potential pass through the double preparation from the right to the left foot. The e. m. f. of the preparation was determined immediately before each double observation by adjusting the galvanometer to zero with the help of the compensator. Two assistants — one for the right and one for the left muscle — watch for the first perceptible contraction in each muscle.

Series I. Direction of the shocks left—right. E. m. f. of preparation + electrodes at 13.47 hrs. = 1.1 mv.

Threshold for *right* muscle at 13.48 hrs. = 0.097 microamps.

» » *left* » » 13.49 hrs. = 0.157 microamps.

Series II. Direction of the shocks right—left. E. m. f. at 13.50 hrs. = 9.1 mv.

Threshold for *left* muscle at 13.51 hrs. = 0.187 microamps.

» » *right* » » 13.52 hrs. = 0.210 microamps.

Series III. Direction of the shocks left—right. E. m. f. at 13.52 hrs. = 11.4 mv.

Threshold for *right* muscle at 13.54 hrs. = 0.120 microamps.

» » *left* » » 13.55 hrs. = 0.191 microamps.

Series IV. Direction of the shocks right—left. E. m. f. at 13.55.5 hrs. = 12.5 mv.

Threshold for *left* muscle at 13.56.5 hrs. = 0.176 microamps.

» » *right* » » 13.57.5 hrs. = 0.210 microamps.

Direct stimulation of the nerve plexuses with controlled current from the generator at 14.01 hrs. immediately after opening the moist chamber, showed that the plexuses on both sides were fully sensitive to stimuli throughout their length. The length of the isolated plexus was found to be on the right side 15.0 mm., on the left side 15.2 mm.

The average threshold values are:

musculopetal direction of the shocks (mp) ...	0.145 microamps.
musculofugal » » » » (mf) ...	0.192 »

The ratio mf : mp is therefore as 1.324 : 1.

From this we conclude that the threshold is lowest when the shocks of negative potential induce a movement of anions in the axis cylinder towards the muscle. It should be noted that this experiment shows very clearly that the threshold was lower in the nerve which was further from the cathode than in the one that was nearer to it. GILDEMEISTER, who reported the determinations of 1909 in *Hermanns Jahresberichte* (for the year 1909) insinuates that it is self-evident that all stimulus automatically emanates from the cathode even in single shocks. The present experiment, in which no cathode is placed on a nerve, shows, however, that the direction of the flow of current in the nerve may in special cases have more effect upon the threshold level than proximity to the cathode.

Pflüger's rule applies as is well known to the effect of the direction of flow upon the reaction of the muscle to bipolar stimulus of its nerve on closing or breaking a constant current. This rule cannot be applied to momentary currents, which constitute the type of electrical stimulus that corresponds to the physiological action of white nerve fibers. It cannot therefore provide any insight into the biological changes associated with a natural nerve impulse or into the problem of whether or not the direction of the momentary current is of any significance in the induction of an experimental nerve impulse. Elucidation of this problem required an all-round investigation with single unidirectional shocks of electricity of known sign, and this was in fact carried out in the work of 1909 quoted previously.

Symmetrical double preparations possess the great advantage over unilateral preparations that they provide their own controls inasmuch as the complex of phenomena observed — as the observations just described show — develops into its mirror image when the direction of the current is reversed. It was found, however, that complete or even sufficient symmetry in excitability in bilateral preparations is the exception rather than the rule and that even a small degree of asymmetry causes the threshold to be lower in the more excitable half of the preparation whichever way the current is flowing. The bilateral preparation was not suited therefore to provide sufficient material for a satisfactory

Table II.

1			2			3			4			5		
Direction of pulses	Time	Threshold in units $\times 10^{-9}$ ampère	Direction of electric im- pulses	Time	Threshold in units $\times 10^{-9}$ ampère	Direction of electric im- pulses	Time	Threshold in units $\times 10^{-9}$ ampère	Direction of electric im- pulses	Time	Threshold in units $\times 10^{-9}$ ampère	Direction of electric im- pulses	Time	Threshold in units $\times 10^{-9}$ ampère
+	12.15	255	+	14.06	337	+	11.34.5	217	+	13.42	202	+	13.36	442
+	12.16	210	+	14.07	285	+	11.35	202	+	13.43	187	+	13.37	427
+	12.17	232	+	Pause		+	Pause		+	13.43.5	210	+	13.37.5	438
+	12.18	210	+	14.12	337	+	11.50	232	+	13.44	210	+	13.38	412
+	12.19	225	+	14.12.5	315	+	11.50.5	217	+	13.44.5	210			
+	12.21	210	+	14.13	333	+	11.51	240	+	13.45	210			
+	12.21.5	225	+	14.14	307	+	11.52	210	+	Pause	202			
	Pause								+	13.49	202			
+	12.30	225							+	13.50	202			
+	12.30.5	232												
+	Pause													
+	12.35	225												
+	12.36	232												
+	Pause													
+	12.46	232												
+	12.47	232												
+	12.56	225												
+	12.57	217												
+	Pause													
+	13.10	210												
EMF var. = 8-11.5-7 millivolt Lp = 20 mm			EMF var. = 9-5.5-15 millivolt Lp = 12 mm			EMF var. = 48-37.5 millivolt Lp = 8 mm			EMF var. = 27.5-17 millivolt Lp = 10 mm			EMF var. = 28-26 millivolt Lp = 11 mm		

Table III.

6			7			8			9			10		
Direction of electric im-pulses	Time	Threshold in units 1×10^{-9} ampère	Direction of electric im-pulses	Time	Threshold in units 1×10^{-9} ampère	Direction of electric im-pulses	Time	Threshold in units 1×10^{-9} ampère	Direction of electric im-pulses	Time	Threshold in units 1×10^{-9} ampère	Direction of electric im-pulses	Time	Threshold in units 1×10^{-9} ampère
↑	14.15	210	↑	14.25	202	↑	13.43	615	↑	13.10	413	↑	14.41	712
↑	14.15.5	172	↑	14.25.5	195	↑	13.44	390	↑	13.11	322	↑	14.44	637
↑	14.16	210	↑	14.26	202	↑	13.45	562	↑	13.11.5	322	↑	14.45	735
↑	14.16.5	172	↑	14.26.5	210	↑	13.46	375	↑	13.12	322	↑	14.46	562
↑	14.17	210	↑	Pause		↑	13.47	540	↑	13.12.5	315	↑	14.47	690
↑	14.17.5	172	↑	14.32	195	↑	13.48	375	↑	13.13	322	↑	14.47.5	607
↑	Pause		↑	14.32.5	195	↑	13.49	532	↑	Pause		↑	14.49	645
↑	14.21	202	↑	EMF var. = 92—78.5 millivolt		↑	13.50	375	↑	13.19	315	↑	14.49.5	600
↑	14.21.5	180	↑	Lp = 12 mm		↑	Pause	442	↑	13.20	300	↑	14.50	622
↑	14.22	202	↑			↑	14.00	382	↑			↑	14.51	577
↑	14.23	172	↑			↑	14.01		↑			↑		
EMF var. = 19—19.5 millivolt Lp = 11 mm			EMF var. = 22—2.5 millivolt Lp = 13 mm			EMF var. = 37.5—19.5 millivolt Lp = 9 mm			EMF var. = 47—27.5 millivolt Lp = 11 mm					

EMF var. = The electromotive force in millivolt of the neuron preparation + electrodes varied during the threshold determinations between

Lp = Length of the sciatic plexus lying bare in the preparation.

solution of the problem; circumstances necessitated for this reason the use of unilateral preparations.

The results of ten experiments carried out in November and December 1945 on unilateral preparations are shown in tabular form in Tables II and III.

The unilateral experiments comprised 42 double observations, which yielded the following statistical results: the average threshold current was 0.342 microamps. when flowing away from the muscle (mf) and 0.299 microamps. when flowing towards it (mp).

The relationship between these two is expressed as $\frac{mf}{mp}$ i. e. $\frac{1.144}{1}$.

The mean of the differences in the total number of double observations is 0.0426 microamps., the standard error of the mean being 0.00875 microamps. It is therefore statistically proven that in the experiments periodic shocks of negative potential stimulate the muscle more when flowing towards it than when flowing away from it, but the relationship between the two threshold values

i. e. $\frac{mf}{mp}$ is found to be much smaller than in experiments with isolated single shocks.

Discussion and Interpretation.

The changes within the nerve fibers which may explain the quantitative differences between the results of the experiments reported in this paper, in which repetitive shocks were employed, and of those obtained in 1909, when isolated shocks were used, can only be discussed after certain theoretical assumptions have been made.

The writers base their argumentation upon the conception of the mode of action of the white nerve fibers which G. F. GÖTHLIN has expounded in several papers and which is to a large extent based upon his own experimental observations. Since, however, neither his views nor the observations upon which they are based are mentioned in the textbooks of physiology it is necessary to explain certain details which are essential to a comprehension of the results reported in this paper.

Struck by a statement of HERMANN (1898 pp. 282—283; 1905, p. 127, foot-note) to the effect that if one applied his "*Kernleiter*" theory to the individual nerve fiber then the myelin sheath must be included in the "*Kern*" and only the connective tissue sheaths

(including Schwann's sheath) could be regarded as the covering, GÖTHLIN carried out in 1902 (pp. 155—161) an investigation into the electrical conductivity of the myelin sheath. The material he used consisted of punched out blocks of the corpus callosum of cattle in which the fibers run parallel but are not enclosed either in Schwann's sheaths or in lamellae of connective tissue. The whole problem is in this material much simpler than in peripheral nerves.

A specially designed resistance vessel of glass (length and width 1 cm., height 0.5 cm.) with electro-plated platinum electrodes was packed with lamellae of corpus callosum cut out with a double knife parallel with the fibers. This vessel was placed in a larger water-tight vessel which could be immersed in a water bath. At body temperature (38°C) the electrical resistance of the block per cc. was determined in the longitudinal direction of the fibers. With a Cohn electrode, the electrolytic conductivity of the vitreous humour of an eye from the same animal was determined after it had been proved that it corresponded (within the margin of error of the method of measurement) with that of the cerebrospinal fluid (CSF) of the same animal.

In order to check the following calculations, it is necessary to know certain dimensions determined by G. F. G. He found by examination of sections $2\ \mu$ thick through the corpus callosum, which had been fixed in osmic acid and cut transversely to the direction of the fibers, that the average thickness of a nerve fiber in that region was $4.34\ \mu$ and the thickness of its myelin sheath $0.9\ \mu$; its axis cylinder had a diameter of $2.54\ \mu$, and a cross-sectional area of $5.1 \times 10^{-8}\text{ cm}^2$.

Assuming that the corpus callosum consists partly of matter of the conductivity of cerebrospinal fluid and partly of non-conductive matter it was possible, by measuring the resistance of the corpus callosum, to calculate how much of the transverse section consisted of *non-conductive matter*. It was found on passing currents through in the direction of the fibers that in seven experiments on an equal number of animals the average proportion of non-conductive matter was 91.4 % of the cross-sectional area. The proportion of the cross-sectional area which would be occupied by the myelin sheaths of nerve fibers in the corpus callosum at an average fiber diameter of $4.34\ \mu$, was only 59.6 %, even assuming that the fibers were so tightly packed that their myelin sheaths were in contact.

On the same occasion, the dielectric constant was determined by Nernst's method (1901, pp. 137—145; 1910, pp. 90—96) both of a lipoid extract of the corpus callosum — after the extract had been dehydrated in a desiccator — and of neurokeratin. The dielectric constant of both these constituents of the myelin sheath was found to lie below 4.0. Combining this result with the theoretical considerations of NERNST (1894) the author inferred that of the structures of the white nerve fiber the myelin sheath must have more insulating properties than any other and must have played the rôle of a non-conducting material in relation to the weak electromotive forces required for his measurement of the resistance in the corpus callosum.

Thus, the myelin sheath does not belong to the *Kern* of the *Kernleiter* but constitutes instead the insulating covering of the white nerve fiber. In order to calculate its inductive capacity the dielectric constant of the myelin sheath must be known. When the work was resumed somewhat later (1907, pp. 101—118; 1910, pp. 90—111), the latter requirement was in so far fulfilled as the dielectric constant of an extract containing the lipoids of corpus callosum and saturated with aqueous vapour at body temperature, was determined by Drude's method (1902) with electric waves and found to be 10.5. The inductive capacity (c) of the myelin sheath per unit length could then be calculated from the formula for cylinder condensers. This was done for an average sized nerve fiber in the corpus callosum. The axis cylinder's resistance in ohms (ω) per unit length was also calculated, assuming that its conductivity was the same as that of the cerebro-spinal fluid, and found to be in round figures one thousand megohms per cm.

Further it was assumed that the frequency (n) of the elementary impulses in this fiber from the corpus callosum corresponded with the frequency ($n = 49$) just determined by PIPER (1908) of action waves in a voluntary muscle of a human being. After making these determinations and assumptions, the rate of conduction (v) in a nerve fiber of this type was calculated (GÖTHLIN 1910, p. 133) in accordance with THOMSON'S (1856) cable formula

$$v = 2 \sqrt{\frac{\pi \cdot n}{c \cdot \omega}}.$$
 In this way, a rate of conduction of 2.4 m./sec. was arrived at for the nerve fiber in question. It had never previously been possible to use THOMSON'S formula to calculate the rate of conduction in a nerve fiber, since no experimental investigations

into the physical quantities c and ω had been carried out up to this date.

The calculated rate of conduction was thus of the same order of magnitude (m./sec.) as the rate of conduction in white nerve fibers arrived at by direct measurement. This is more than can be said even today of any other hypothesis regarding the conduction in white nerve fibers based upon real physico-chemical measurements (GÖTHLIN 1917, 1939). In view of this correspondence, GÖTHLIN (1910 p. 141) suggested as a working hypothesis that the white nerve fiber is an elementary cable in the form of a cylindrical condenser in which the axial conductor is the axis cylinder with the conducting power of an electrolyte while the myelin sheath is the insulating layer which separates the electrolyte in the axis cylinder from the electrolyte surrounding the fiber. This hypothesis leads to the conclusion that coarse nerve fibers must conduct more rapidly than fine ones and it is therefore strongly supported by the results later obtained by ERLANGER and GASSER (1927, 1937.)

It is well known that the myelin sheath is interrupted at every node of RANVIER, and this gap is occupied by RANVIER's "constricting band" which in polarised light and in fact in general possesses very different properties from myelin. Proof of less isolating properties of these constricting bands is provided by experiments carried out by KUBO and ONO and described by KATO (1934, pp. 68—76). Current transmitted by microelectrodes was found in these experiments to have a far greater excitatory action when the electrode was placed on a node of RANVIER than when it was placed upon an internode. This finding justifies the assumption that the constricting bands possess a certain conductive capacity for currents of low e. m. f. In this work it has therefore been assumed that branches of the exciting current penetrate to and emerge from the axis cylinder at the nodes of RANVIER and that they pass through the axis cylinder between these two points, though at a much lower density than in the lymph spaces which surround the nerve fiber.

It is now generally recognised that the action current is able during its passage to regenerate its own charge by inducing a chemical reaction by virtue of its own e. m. f. There are, however, different opinions as to the mechanism whereby this reaction is brought about [v. i. a. CREMER (1909, 1929), GÖTHLIN (1910, 1917), BROEMSER (1927), ADRIAN (1931), HILL (1932), v. MURALT (1945)].

GÖTHLIN (1910, p. 141, and 1917, pp. 511—513) assumes that the neurofibrils consist of an axis of a dielectric proteotrop (cp. GÖTHLIN 1913, plate 3, fig. 6) material and a chemically highly reactive superficial layer with electrolytic conductivity, and that in the axis cylinder, at the interface between these two layers, the action potential brings about a dielectric polarisation of the fibrils analogous to that in the phenomenon known as *electrostenolysis* (BRAUN 1891) and by this means induces in the superficial layer of the fibrils a chemical reaction creating an excess of unstable anions.

The CO_3 ions may play a rôle in this reaction, as the formation of CO_2 in nerves is increased when they are transmitting impulses (TASHIRO, 1913). The CO_3 ion *in statu nascendi* in the presence of oxygen would have a very different effect upon the excitatory state in the axis cylinder from that of an existing stable system of one CO_3 ion and two H ions.

When a shock of negative potential is sent through the axis cylinder the primary effect in the latter is a propulsion of its anions from the negative pole towards the grounded lead of the system. In addition it is assumed — in conformity to the supposed course of events in the action potential wave — that every shock of negative potential induces by polarisation at the interface of the fibrils a chemical reaction which results in the production of a temporary excess of anions, possibly including CO_3 ions.

Although the changes which a negative shock induces in the axis cylinders may be assumed to be in many respects similar in kind to those which occur in the region of an action potential wave, there is the important difference that the former changes are induced *simultaneously* in all those parts of the neuron which lie within the exposed part of the sciatic plexus.

With regard to *isolated* shocks of negative potential, earlier experiments had shown that the threshold of the muscle is greatly affected by their direction and is reached, if the shock propels the axis cylinder's anions towards the effector organ, at about half the potential which is required when the shock has the opposite direction. With the repetitive stimuli of negative potential which are employed in the present investigation at a frequency so chosen that — judging by K. LUCAS' and E. D. ADRIAN's experiments (1917, p. 35) — each shock reaches the preparation in the beginning of the supernormal phase following the previous one,

the mean relationship $\frac{1.144}{1}$ between the threshold values of the

musculofugal and the musculopetal propagation is much lower. This seems to indicate that the repetition of the shocks, at least at the chosen frequency, brings about an increase in the chemical reactions not only absolutely but also in proportion to the propulsive effect and that *this occurs irrespective of whether the shocks drive the anions to or from the end organ.*

At all events the differences in influence of the direction of shocks of negative potential on the threshold values which have appeared when examining one time with single shocks, the other time with a physiologically adjusted series of shocks, prove that there are two factors which determine the effect of the electrical currents upon the nerve and so indirectly upon the muscle. The difference in excitatory effect between musculopetal and musculofugal shocks of negative potential the authors regard as evidence of a direction factor in the electric excitation process — a factor which is positively excitatory when the anions in the axis cylinders are propelled by the electric forces in the direction of the physiological end-station but negative, and inhibitory, if the electric forces induce propulsion of the anions in the axis cylinders in the opposite direction. A second factor is the polarisation process which induces chemical reactions in the axis cylinders and in this way brings about a transitory excess of unstable anions corresponding to every single shock.

On the whole the experiments show that the relative influence of these two factors is such that the direction and ion propulsion factor is predominant when isolated shocks are administered while the polarisation and chemical reaction factor is proportionally more effective when repeated stimuli are applied at physiological intervals.

If one assumes — as the relative levels of the thresholds — $E \uparrow$ and $-E \downarrow$ in the experiment with isolated shocks (cf. Table 1) suggest — that the direction factor *inhibits* in the case of musculofugal shocks *to the same degree* as it *enhances* the excitatory effect in musculopetal shocks, then one can also calculate numerically the proportion of the excitatory effect for which the direction factor and the ion propulsion factor are responsible when the current flows musculopetally. On the above assumption the influence of one direction should amount to half the difference between the threshold values for musculofugal and musculopetal shocks. From this one can calculate the direct part (DF) played by propulsion of ions in the total excitatory effect of a musculopetal current. If this method of calculation is applied the following figures are obtained.

In the case of isolated shocks on the lines of the experiment described in Table 1, if the average of the three threshold values for $-E \downarrow$ is taken as the basis of the calculation, then $DF = \frac{0.5(676-305)}{305} = 60.8\%$, whereas 39.2% of the excitatory effect is due to secondary polarisation reactions. In the experiments, in which periodic shocks of negative potential were administered to a neuron preparation, the excitatory effect D_1F_1 of a propulsion of ions induced in the axis cylinders of the primarily excited parts of the neurons and propagated as such to lower regions, amounts to $\frac{0.5(341.5-298.9)}{298.9} = 7.1\%$, whereas 92.9% of the excitatory effect is due to secondary polarisation reactions.

It is probable that the length of the portion of nerve fiber that is effectively excited will have some influence on the results (cp. RUSHTON 1927), but it did not belong to the object of the writers to take up this question. In 9 out of 10 experiments the exposed length of the neurons varied from 8 to 13 mm.

It remains to remark that if an apparatus could be constructed which would enable one to investigate *on the same preparation* both the effect of single shocks and the effect of different series of shocks of various frequencies, the relative proportions of the two factors would be more satisfactorily ascertained and it would be possible to observe transition states from the one extreme to the other by means of the method of analysis described above.

Summary.

1. One of the authors (G. F. G.) in an earlier investigation (1907, 1909) established that with sciatic-gastrocnemius preparations, on unipolar application to the nerve of single shocks of negative potential, the threshold of excitation of the nerve for a *musculopetal* direction of the shock was attained at about half the potential which was necessary on applying a similar shock in *musculofugal* direction (cp. Table 1).

2. In the present series of experiments, surviving peripheral motor neurons of frogs are used to determine the threshold of the nerve to shocks of negative potential repeated regularly at a frequency of 75/sec. The impulses are generated by an impulse

generator (Fig. 3) and their wave form is shown in Fig. 4 and 5. The threshold current is recorded by a sensitive galvanometer. — In this series it is found in 42 double observations that the mean value of the threshold in the case of musculofugal shocks (mf) is 0.342 microamps., while in the case of musculopetal shocks (mp) it is 0.299 microamps, *i. e.* $\frac{mf}{mp} = \frac{0.342}{0.299}$. The mean difference between the two values in the 42 double observations is 0.0426. The standard error of the mean is 0.00875. It is thus statistically established in this series too that *musculopetal shocks of negative potential have greater excitatory effect than musculofugal.*

3. From the very different values of the quotient $\frac{mf}{mp}$ in the two series — for single shocks about $\frac{2}{1}$ and for regularly repeated shocks of a frequency of 75/sec. $\frac{1.144}{1}$ it is inferred that two factors are concerned in the excitatory effect of the shock upon the nerve and indirectly upon the muscle, viz.

i. a *direction factor* DF dependent on the direction in which the shock propels the anions in the axis cylinder, inasmuch as their propulsion *towards* the muscle *enhances* but their propulsion away from it *diminishes* the excitatory effect, and

ii. a *reaction factor* RF dependent on the size of regularly repeated but transient aggregations of anions which the shocks induce in the axis cylinder by means of dielectric polarisation. The probable mechanism of this process is dealt with under the heading: "Discussion". — The experiments show that the factor DF is predominant when single shocks are used while the factor RF is preponderant when regularly repeated shocks of optimum frequency are administered.

4. On a certain assumption, the validity of which is argued under "Discussion", numerical values of DF and RF can be given, as follows: In the experiment with *single* musculopetal shocks of negative potential DF amounts to 60.8 % and RF to 39.2 % of the total excitatory effect in the nerve. In the experiments in which *periodic* musculopetal shocks of negative potential were administered to a neuron preparation D_1F_1 amounts to 7.1 % and R_1F_1 to 92.9 % of the total.

It may be assumed that in physiological motor nerve impulses,

the influence of secondary polarisation reactions upon the excitatory effect as to order of magnitude best harmonizes with the experiments with periodic stimulation by negative musculo-petal shocks of optimum frequency.

The Therese and Johan Andersson Fund of the Caroline Institute, Stockholm, has defrayed the expense of building the impulse generator. The authors are indebted to Professor TORSTEN TEORELL for the use of several instruments. Skilfull technical assistance in the experiments was lent by Miss AGNETA LILJEGREN, Cand. Med.

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From the Copenhagen University Institute of General Pathology.

Comparative Tests of the Thiosulphate and Creatinine Clearance in Rabbits and Cats.

By

JENS BING and POUL EFFERSØE.

Received 2 January 1948.

It was demonstrated by GILMAN, PHILIPS and KOELLE (1946) that the clearances of thiosulphate and creatinine are identical in dogs, and this was subsequently verified by PITTS and LOT-SPEICH (1947). Later, in the course of experiments on man, NEWMAN, GILMAN and PHILIPS (1946) and BRUN (1948) showed that the thiosulphate clearance is identical with the inulin clearance, so that we may take it that the thiosulphate clearance in dogs and in man provides a measure of the filtration through the glomeruli. In the following study a comparison is made on rabbits and cats of the creatinine and thiosulphate clearances for the purpose of ascertaining whether the easily determinable thiosulphate clearance can also be utilized in these animals for determining the glomerular filtration.

Analytical Technique, Material and Experimental Method.

The contents of creatinine and thiosulphate in plasma were determined after precipitating the proteins with tungstic acid, one volume of 10 % sodium wolframate, one of 2/3 n sulphuric acid and two of distilled water being added to one volume of plasma, the sediment thereafter being centrifuged off.

The *creatinine* in blood and urine were determined spectrophotometrically² at wave lengths of 530 and 500 respectively according to

¹ The experiments were carried out by means of aid from the "King Christian X. Foundation" and the "Frk. P. A. Brandt Bequest".

² We thank Dr. HERMAN KALCKAR for giving us his kind permission to borrow the Beckman spectrophotometer.

FOLIN's method, including four control samples of known content in each test. Photometry was applied at least one hour and less than seven hours after the addition of the picric acid and alkali, a period within which the values were found to remain constant.

Thiosulphate. The determinations in plasma filtrate and urine were carried out by CLAUS BRUN's method (1948), which briefly consists of diluting the sample with water and then to 3 ml. adding 1 ml. 2*n* hydrochloric acid and one or two drops of 1 % starch solution, followed by titrating with *n*/1000 iodine in potassium iodide solution. The standard used was a *n*/400 sodium-thiosulphate solution stabilized with mercuric cyanide. In the titration of urine and blood from rabbits and cats we found low blank values, for which correction was made.

The *material* comprised 11 full-grown buck rabbits of various breeds and 10 full-grown cats of different breeds and sexes.

The *rabbits* weighed from 2 to 3½ kg. Four were anaesthetized by subcutaneous injection of 0.55—0.6 g. uretan per kg.; the others were not anaesthetized, with the result that anaesthesia was found to have no bearing on the tests. Fifteen to thirty minutes before commencing the experiment about 200 ml. lukewarm water was administered by stomach-tube to ensure ample diuresis.

About ½ g. creatinine per kg. was injected subcutaneously before commencing. In more prolonged experiments it was found necessary to give a second injection later. In all cases at least an hour elapsed after the creatinine injection before the clearance periods began.

The sodium-thiosulphate was injected subcutaneously dissolved in 40—70 ml. water, once or more often during the experiment; here too about an hour was allowed to elapse between injection and the beginning of the clearance periods.

The clearance periods lasted from about 10 to 30 minutes. The clearance values shown are those that were found, without correction for the size of the animal. The catheter remained in position throughout, and the bladder was washed out with saline. The blood samples were taken from the carotid artery.

The *cats* weighed from 2 to 5½ kg. and all were anaesthetized with about 1 g. uretan per kg.

The technique otherwise was the same as in the rabbit tests, except that the catheter was stitched into the exposed vesica, and the urine and lavage water running out of the urethra were collected in a bowl.

In a few cases the thiosulphate was injected into the cats intravenously, 1—4 g. in 5—20 ml. over a period of 3 to 10 minutes. In one experiment where the injection was given rapidly the animal vomited; this had also been observed by the previous investigators (3) after rapid injection.

Results.

Rabbits: A total of 56 simultaneous determinations of the creatinine and thiosulphate clearances were made on 11 rabbits.

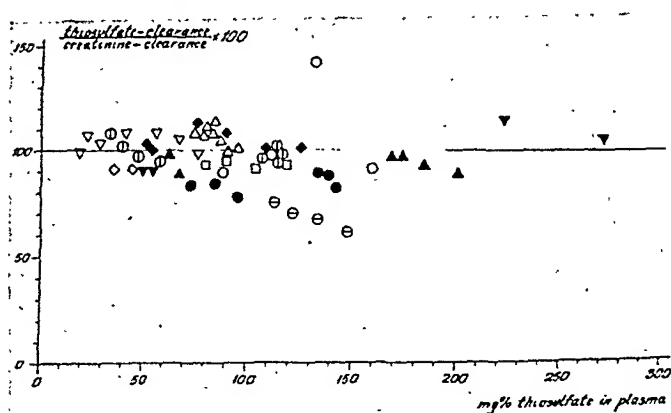


Fig. 1. The figure shows the result of comparative experiments on the creatinine and thiosulphate clearances with varying thiosulphate concentrations in the blood of 11 rabbits. It will be seen that the thiosulphate clearance, expressed as a percentage of the creatinine clearance, in the nine rabbits lies round about 100 % of the value. In two animals somewhat lower values were found. Various symbols are employed for the different animals.

The creatinine concentration index varied between about 8 and 120, and the creatinine clearance between 1.34 and 14.23 ml. per minute.

In fig. 1 the thiosulphate clearance is expressed as a percentage of the creatinine clearance. For two rabbits we found percentages distinctly below 100. If these two are disregarded, the dots of the other nine tests are distributed evenly about the identity line, the average being 99.9. Including the two deviating results the average works out at 95.9.

Table 1.

Example of a rabbit test. Despite varying thiosulphate concentration in the blood and variations in both creatinine concentration index and clearance, there are identical values for thiosulphate and creatinine clearances. Rabbit 129, tested 17-10-47.

Urine period	mg% Thio-sulfate in blood	Creatinine concentration index	Creatinine clearance	Thiosulfate clearance	Thiosulfate-CI.
					Creatinine-CI ¹⁰⁰
1	56.8	42.0	7.0	6.51	93
2	48.4	53.1	8.44	8.19	97
3	40.4	43.7	9.79	9.95	102
4	33.6	47.3	7.43	8.04	108
5	108.8	16.23	5.28	5.05	96
6	114.1	19.35	3.39	3.30	98
7	114.5	26.75	2.82	2.71	97
8	114.1	11.27	2.63	2.65	101

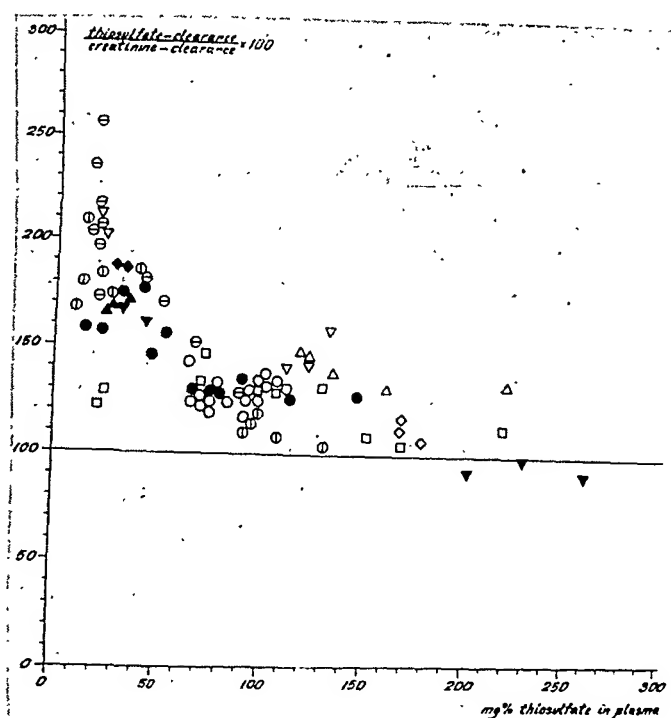


Fig. 2. The figure shows the result of comparative experiments on the creatinine and thiosulphate clearances with varying thiosulphate concentrations in the blood of 11 cats. It will be seen that the thiosulphate clearance, which is expressed as a percentage of the creatinine clearance, with low blood concentrations reaches up over 200% of the creatinine clearance, and that the difference between the two clearances diminishes steadily with an increasing thiosulphate concentration in the blood. Various symbols are employed for the different animals.

Thiosulphate clearance

$\frac{\text{Thiosulphate clearance}}{\text{Creatinine clearance}}$ was thus found to be independent of the thiosulphate concentration in the blood, which varied between 19 and 274 mg %. And, as Table 1 shows, this ratio was found to be independent of variations in the concentration index and the clearance for creatinine within the limits of the test.

Cats: A total of 78 simultaneous determinations of the thiosulphate and creatinine clearances were made on 11 cats.

The creatinine concentration index varied from 30 to 78.9 and the creatinine clearance from 0.17 to 17.5 ml. per min. The plasma creatinine concentration lay between 45 and 8 mg %, and the plasma thiosulphate concentration between 9 and 263 mg %. The determinations were made partly with a falling and partly with a rising plasma thiosulphate concentration.

Fig. 2, on which all determinations from the 11 cats are plotted

Table 2.

Example of a cat test. The $\frac{\text{thiosulphate clearance}}{\text{creatinine clearance}}$ ratio is seen to rise with a falling thiosulphate concentration in the blood, whereas in this test, where the diuresis was varied by intravenous sulphate injections, it was found to be independent of the variation in the creatinine concentration index and the creatinine clearance. Cat, tested 11-11-47.

Urine period	mg% Thio-sulfate in blood	Creatinine concentration index	Creatinine clearance	Thiosulfate clearance	$\frac{\text{Thiosulfate-Cl.}}{\text{Creatinine-Cl.}} \times 100$
1 ...	95.7	22.7	9.08	11.60	129
2 ...	67	28.25	7.06	10.75	152
3 ...	50.6	38.3	7.66	13.08	171
4 ...	40.7	42.0	6.30	11.55	183
5 ...	19.2	39.0	4.64	11.88	256
6 ...	16.2	10.7	6.84	16.10	235
7 ...	16.6	4.82	1.775	3.63	205
8 ...	19.8	12.3	0.171	0.293	173
9 ...	19.7	6.93	0.533	1.11	208
10 ...	19.2	4.27	1.33	2.62	197
11 ...	19.2	3.0	0.173	0.374	216

(without correction for the different sizes of the animals) shows that the thiosulphate clearance of cats with a low thiosulphate concentration in plasma is higher — up to twice as high — than the creatinine clearance. With an increasing plasma thiosulphate concentration this difference is diminished (in values of over about 200 mg % the difference presumably lies within the experimental error). As the injection of thiosulphate has a diuretic effect there was in most cases inverse proportionality between the blood thiosulphate concentration and the creatinine concentration index; but in one test where the degree of the urine concentration was varied by intravenous injections of sulphate (Table 2), the relation between thiosulphate and creatinine clearance was found to vary only with the thiosulphate concentration in the blood, whereas it was unaffected by large variations in the creatinine concentration index and the creatinine clearance.

Discussion and Conclusions.

Rabbits. Identity having been found between the creatinine and thiosulphate clearances in rabbits with plasma thiosulphate concentrations between 19 and 274 mg % and with greatly varying creatinine concentration index and clearance, the thiosulphate

clearance within the said limits is just as good a measure of the renal filtration as the creatinine clearance. In two of the eleven tests, however, the thiosulphate clearance was found to be slightly lower than the creatinine clearance. There is no saying whether this was due to experimental error or there was a reabsorption of thiosulphate in the tubuli.

Cats. No similar identity between the creatinine and thiosulphate clearances was found in the cats such as that observed in man, dogs and rabbits.

At very high thiosulphate concentrations in the blood the two values seem to coincide, it is true; but with falling blood concentrations the thiosulphate-creatinine ratio rises, as will be seen from fig. 2.

The difference observed between cats on the one hand and man, dogs and rabbits on the other, is easiest to explain by assuming that cats are able to secrete thiosulphate, for it is characteristic of secretion substances that with increasing plasma concentration comes self-depression with a fall in the clearance, which thereby approaches the value of the filtration.

Summary.

1. In *rabbits*, simultaneous determinations have demonstrated identity between creatinine and thiosulphate clearances in 9 tests though for two other animals the values for the thiosulphate clearance were slightly lower than for the creatinine clearance.

2. In *cats*, on the other hand, the thiosulphate clearance when the plasma thiosulphate concentrations are low may have a value twice as high as that of the creatinine clearance, whereas with a rising plasma thiosulphate concentration the difference approaches 0. Presumably the explanation is that the cat kidney can secrete thiosulphate.

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On the Blood Supply to the Brain during Acceleration.

By

SVEN ÅKESSON.

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In the past decade the problem of the blood supply to the brain during positive acceleration (from head to seat) has been keenly discussed in aviation medicine. With an acceleration of $+5\text{ g}$ consciousness can generally be maintained for 15 seconds or more (RUFF and STRUGHOLD 1944, McFARLAND 1946), in spite of the fact that the weight of the blood increases 5 times; *i. e.* approaches that of melted iron. The heart would be forced to lift the heavy column of blood from the level of the heart to that of the brain, while, at the same time, the reflux of blood to the heart, and the consequent diastolic filling of the ventricles, is considerably reduced.

A study of a schematic drawing of the circulation through the neck and brain immediately suggests the question whether a siphonic effect is, possibly, in evidence. When hydrostatic pressure reaches such a height as to cause a negative blood pressure in the skull, can it be, then, that the supply of blood to the brain is effectuated by a siphonic function of the blood system, with the carotids as an afferent and the jugular veins as an efferent shank? Supposing that Level I in Fig. 1 would mark a sufficient height of pressure at rest to overcome the resistance in the brain and impart to the blood an adequate velocity, this would mean that at an acceleration of $+5\text{ g}$ it would suffice if the blood were pumped up to Level II ($h/5$). The only increase in cardiac activity required would involve that relatively insignificant component which produces the acceleration of the blood itself. However, in

order to maintain a siphonic effect, the vascular lumina must nowhere collapse under the trials indicated here, thereby breaking the siphonic function. The weak points are the jugular veins and the small vessels within the skull cavity. The siphon theory

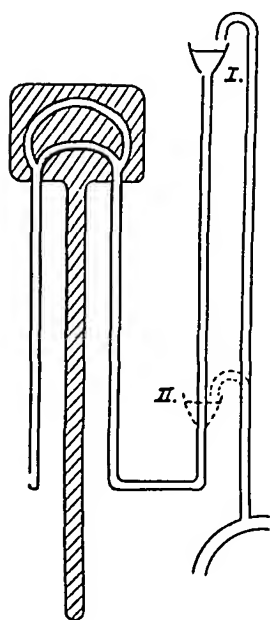


Fig. 1. Schematic drawing of the hemodynamic and the hydrostatic system in the brain. If the hemodynamic system functions like a siphon, the blood at an acceleration of 5 g need only be pumped to position II ($h/5$ of position I).

(v. DIRINGSHOFEN 1937, RANKE 1938) has previously categorically been repudiated for the reason that the jugular veins must give way even at but a slight drawing effect. On the other hand, RANKE (1938), with regard to the small cerebral vessels, has stressed the significance of the interplay between the hemodynamic vascular system and the hydrostatic system of the cerebrospinal fluid, which may conceivably save the cerebral vessels from collapsing.

The purpose of the present investigation is to subject the possibilities of a siphonic effect in the course of acceleration to a close examination. It divides itself into two parts, as follows: How strong is the resistance to pressure of the jugular veins? And what factors will affect the circulation within the skull cavity?

I. Regarding the Resistance of the Jugular Veins to Pressure.

The problem has previously been grappled with simply by freezing rabbits under centrifugation (ROMBERG 1939). Section through the throat showed the veins to have collapsed. Since, at the same time, the cardiac cavities were empty of blood and the blood collected in the abdomen and hind legs, such experiments, of course, explain nothing regarding the resistance of the jugular veins when the heart pumps a reasonably adequate amount of blood. As a matter of fact, clinical experience shows that the jugular veins are fairly resistant to pressure. Queckenstedt's sign is more marked the stronger the pressure on the neck, *i. e.* the more compressed the jugular veins. With a sufficient discharge through the plexus vertebralis, an increased compression should

not, after a complete collapse of the veins, cause any further rise in the pressure of the cerebrospinal fluid.

Attempts at determining this limit have been made in the following manner:

The pressure on the neck has been exerted by means of a tightly fitting blood pressure cuff, 5.5 cm broad, the pressure in it being varied. The pressure of the cerebrospinal fluid has, at the same time, been measured with LAGERGREN's (1937) apparatus. The instrumentarium consists of a capsule, with a mirror for optical registration,

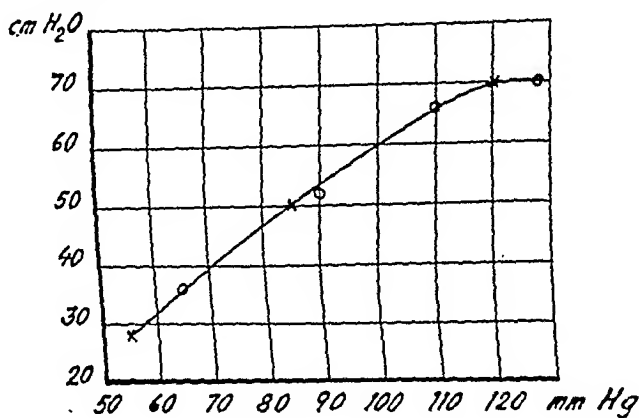


Fig. 2. The rise in the pressure of the cerebrospinal fluid (the ordinate) at varying rises in pressure in a cuff round the neck (the abscissa) for 10 seconds and 15 seconds.

connected to the lumbar needle by means of a rubber tube containing a physiological saline solution. Since the registration at strong compression exceeded the breadth of the film it was, in those instances, recorded directly on a scale. Five patients at the Serafimer Hospital, 28—60 years of age, were examined. The cuff pressure was maintained for 10—15 seconds. Then the rise in the pressure of the cerebrospinal fluid had, as a rule, reached its maximum. Fig. 2, concerning the 60-year-old patient, may serve as an instance. The crosses indicate the pressure of the cerebrospinal fluid after 10, the rings in another series after 15 seconds of stasis. Only at a pressure in the cuff (the abscissa) of > 110 mm Hg, the curve reveals a tendency to turn horizontally. Consequently, a further increase in pressure in the cuff could not be expected to produce any additional effect, *i. e.* the jugular veins had collapsed. In another case the collapse seemed to have occurred at 120 mm Hg. But in the remaining three cases the curve was linear up to 125, 140 and 150 mm Hg, respectively.

If the distance between the basis of the skull and the hydrostatic reference point of the venous system in the upper half of the body, in or just above the heart, be estimated at 15—20 cm, the drawing effect at an acceleration of 5 g would keep within the

limits of 80—100 mm Hg. Keeping in mind the results of the Queckenstedt experiment, it seems unlikely that the veins would collapse. True, the effective pressure on the veins must, obviously, amount to considerably less than the measured pressure in the cuff, but, conversely, the actual drawing pressure in the vessels cannot be 100 per cent effective.

II. The Interplay Between the Pressure in the Blood Vessels and the Cerebrospinal Fluid in the Skull Cavity.

Together with the dural sac around the medulla spinalis the skull constitutes a firm capsule. Yet, the dural sac is not altogether inadaptible. Because of this, the pressure of the cerebrospinal fluid in an erect position is negative in the cranial cavity. As shown by ANTONI (1932) the zero point, *i. e.* the hydrostatic reference point, is situated some centimetres below the occipital foramen. Owing to its increased weight under positive acceleration, the cerebrospinal fluid would be forced down into the dural sac, thus causing a further fall in the pressure within the cranial cavity. It is, however, hardly probable that such a reduction in pressure would be sufficient to balance the falling blood pressure in the small vessels. In all likelihood, other protective mechanisms come into operation. In experiments on cat, FOG (1933) has shown that but a moderate rise in the pressure of the cerebrospinal fluid (not affecting the blood pressure in the large arteries) causes a dilatation of the pial arteries. Inter alia, at bleeding experiments (1937, 1939), a corresponding dilatation sets in (though only after an initial, probably passive reduction in calibre), a fact suggesting that not the absolute pressure of the cerebrospinal fluid but the interrelation between this pressure and that of the blood constitutes the eliciting factor in the mechanism. The very fact that children do not lose consciousness when screaming long and persistently indicates the probability of the existence of such a mechanism also in Man. As shown by HAMILTON and his collaborators (1936, 1944), among others, the pressure of the cerebrospinal fluid rises parallel to the increase in pressure in the thorax and can, in the course of screaming, become considerable. The intrathoracic pressure is rapidly, and practically without being diminished, conveyed to the small cerebral vessels. The simultaneous rise in pressure in the large intrathoracic arteries must necessarily be inadequate to compen-

sate for the increased extravascular pressure in the cranial cavity, considering that the arterial pressure is markedly reduced in the arterioles.

It is difficult to observe directly the pial arteries in Man. However, the problem can be approached by a detour. When the pressure of the cerebrospinal fluid is increased above the probable rest pressure in the cerebral capillaries (the capillary pressure must, reasonably, keep around the sum of the colloid-osmotic pressure of the blood and the pressure of the cerebrospinal fluid in order to ensure an optimal exchange of fluid between blood and tissue, *i. e.* amount to approximately 35 ± 10 mm Hg in a supine position), either the capillaries must collapse, involving in the first place the loss of consciousness, or the pressure must in some way be increased in the capillaries. This rise in the pressure can be achieved via FOG's previously mentioned mechanism, or by increasing the pressure in the arteries. CUSHING (2), as early as in 1901, demonstrated on dogs the fact that an experimental rise in the pressure of the cerebrospinal fluid leads to a pronounced general rise in the blood pressure. However, this does not set in until the pressure in the cerebrospinal fluid reaches an extent approaching that of the large arteries. (Cp. further investigations by FREEMAN and JEFFERS (1939) and FORSTER (1943).)

After a few initial experiments, in which the pressure in the lumbar fluid was increased by raising a level bulb, containing a physiological saline solution, connected with the lumbar needle under continuous blood pressure controls, an attempt was made at double puncture according to the following process.

The test person was a 26-year-old seaman who was being treated at the Nerve Clinic for a suspected medullary tumor, necessitating double puncture *acc. LAGERGREN*. After registration of the pressure in the cisterna magna and in the lumbar canal, 6 cc were drained from either needle. The pressure then equalled 7 cm H₂O. A straight tube was now applied for pressure registration at the suboccipital needle and the level bulb was coupled to the lumbar needle. The investigation record is illustrated in Fig. 3. It was found that the pressure in the cisterna magna responded but sluggishly to elevations of the level bulb (cp. investigations by WOLFF and collaborators 1941, 1943). Thus, no immediate conclusions can be drawn from the height of the level bulb regarding the pressure in the cranial cavity. The pressure in the cisterna magna was gradually raised to 66 cm of water above the initial pressure, *i. e.* an overpressure of > 40 mm Hg. The blood pressure in the arm, measured in the ordinary (non-surgical) way, rose only by 5 mm Hg, while the pulse frequency increased from 60—80. The patient felt well throughout, but towards the end

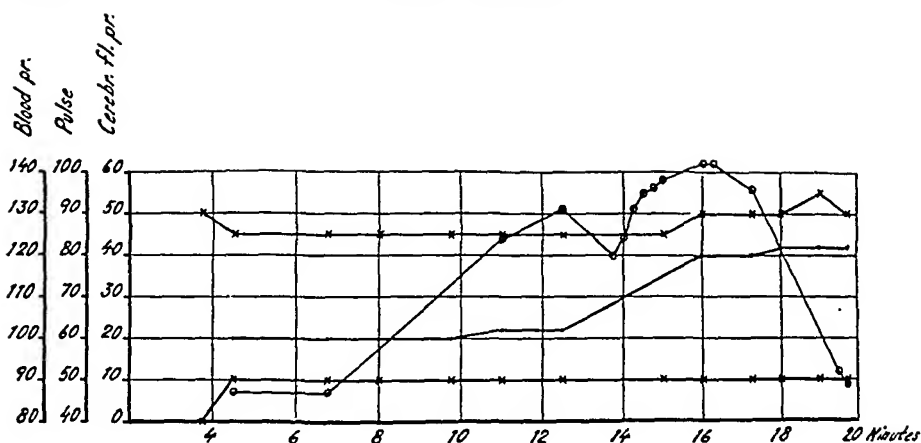


Fig. 3. The interrelation of the blood pressure \times — \times — \times and the pulse \bullet — \bullet — \bullet under artificial rise in pressure \circ — \circ — \circ in the cranial cavity.

of the examination he complained of an unpleasant sensation in the back.

Since the pressure of the cerebrospinal fluid increased considerably above an acceptable rest pressure in the cerebral capillaries while the pressure in art. brachialis remained practically unchanged, it is necessary in this instance to presuppose that Fog's mechanism, or some similar one, has been in action.

During the preliminary experiments the systolic blood pressure in one case rose by 10 mm Hg when the level bulb was lifted by 40 cm. In another case, no increase occurred at an elevation of 50 cm. In both instances, the level bulb was kept elevated for such a long time that the pressure balance had probably had time to take place.

Summing up, it will seem likely that the jugular veins do not collapse at the drawing effect that may occur in these vessels at an acceleration of < 5 g. Further, it appears possible that the physical play of balance between the hemodynamic and hydrostatic system in the brain, together with Fog's mechanism, might simultaneously prevent a collapse of the small vessels in the brain. In these circumstances, it would be conceivable that the circulation in the brain was rendered possible (or at any rate facilitated) through a siphonic effect, thus being maintained as long as the reflux of blood to the heart is adequate.

In the summer of 1947, Wood and collaborators published their results on this subject. They were in a position to show that under positive acceleration the blood pressure will, to begin with, fall but that as soon as after 5—7 seconds it again rises to values

considerably above the rest value, this in a rather surprising manner.

As far as known, no similar reaction has ever been observed in arterial orthostatic anemia. Not unlikely, this is a manifestation of CUSHING's mechanism at a strong overpressure in the cerebrospinal fluid, being the last line of defence of the cerebral circulation, mobilized when the play of balance between the blood and the cerebrospinal fluid and Fog's reflex have proved insufficient. Finally, it should be noted that the rise in pressure ascertained by WOOD seems necessary to explain the resistance of the eye to black-out, since the eye is situated outside the cranial cavity.

Summary.

The possibility that the blood circulation in the brain, in an erect position, particularly under the influence of higher degrees of positive acceleration, is facilitated by a siphonic effect (fig. 1) is discussed. A prerequisite for this is that the vessels do not close at any point of the siphon. The weak points are the jugular veins and the small vessels in the brain. Direct measurements of the pressure of the cerebrospinal fluid during compression tests on the neck show that the jugular veins are far more pressure-resistant than was earlier believed. It seems plausible that the small vessels in the brain are, in the first place, protected from collapse by the hydrostatic equilibrium between the blood and the cerebrospinal fluid. In all probability, a local artery-dilating mechanism, at imminent over-pressure in the cerebrospinal fluid, will contribute towards keeping the vessels open. It has been shown in Man that the pressure in the cerebrospinal fluid can be raised to degrees above the probable pressure in the cerebral capillaries without loss of consciousness and without any significant increase of the blood pressure.

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The Phosphate Metabolism in the Hypophyseal-Diencephalic System and Ovaries of Rats Determined by Means of P^{32} .

By

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The studies which CAMUS and ROUSSY (1920), BAILEY and BREMER (1921), SMITH (1926), BIGGERT and ALEXANDER (1930), BUSTAMANTE, SPATZ and WEISSCHEDEL (1942), and others, have made on rats, rabbits, and dogs showed that damage to the diencephalon may induce atrophy of the genital organs. DEY (1942, 1943) wrote that these changes do not occur unless the median eminence is damaged.

HARRIS (1927) and HATERIUS (1937) found that electrical stimulation of a certain area within the diencephalon induces ovulation in rabbits. According to WESTMAN and JACOBSON (1937) this effect is not produced after transection of the pituitary stalk. WESTMAN, JACOBSON and HILLARP (1943) found that the rhythm of the reproductive cycle is modified in rats and rabbits after this operation. UOTILA (1940) did not observe these changes after transection of the pituitary stalk in rats.

In the light of these conflicting reports it is obviously desirable to obtain additional information as to the importance of the intact connection between the diencephalon and the pituitary in the production of gonadotropic hormones in the adenohypophysis. Such a study, however, presents great difficulties. In operations on the pituitary stalk there is danger of inadvertent damage to the adjacent organs and of disturbance of the blood supply. It is at present difficult to determine the exact significance of either.

In order to get a better idea of the function of the hypophyseal-diencephalic system we have recently made experiments with radioactive isotopes (BORELL, WESTMAN and ÖRSTRÖM 1947). This method has the advantage that one escapes performing an operation. The experiments were made on rabbits in which mating induces ovulation. In the tuber cinereum and adenohypophysis there is, immediately after coitus, a marked increase not only in the turnover of the radioactive phosphate P^{32} , but also in the formation of the phosphate esters which appear in the carbohydrate metabolism. With this technique it was possible to demonstrate that similar metabolic changes occur in the tuber cinereum and adenohypophysis at such time as the incretion of gonadotropin in sufficient quantities to induce ovulation has set in. Against this background the hypophyseal and diencephalic systems seems to constitute a functional unity. We were, therefore, interested in making similar studies on spontaneously ovulating animals to determine whether the phosphate metabolism in the diencephalon, adenohypophysis and ovaries is modified during the phases of the reproductive cycle. The experiments described in the following were made on rats.

Methods and Material.

81 rats of our own breed weighing between 135 and 295 g. were used. Their approximate mean weight was 200 g. In order to ascertain the phase of the reproductive cycle and whether it was regular or not the vaginal smear of each animal was studied for at least three weeks before the beginning of each experiment. The animals were divided into four groups depending on whether they were in prooestrus, oestrus metoestrus or dioestrus. Forty minutes after the intraperitoneal injection of 0.02 mC P^{32} as free phosphate dissolved in 5 per cent glucose the animals were sacrificed by decapitation. The tuber cinereum, adenohypophysis, ovaries, cerebellum and blood were first combusted with H_2SO_4 and then their total contents of both phosphate and radioactivity determined. The total phosphate content was determined by the routine colorimetric method, and the radioactivity measured with the aid of the Geiger-Mueller-counter (for details of these methods see BORELL and ÖRSTRÖM, 1945—1947, and BORELL, WESTMAN and ÖRSTRÖM, 1947).

Owing to the fact that the phosphate content of some of these organs is very small, the organs of two and sometimes even of three animals were united to obtain measurable amounts of phosphate. The values obtained are expressed in terms of *specific activity*, i. e. number of impulses per σ P and of *relative specific activity*, where

both the cerebellum and blood have been used as standards. The specific activities measured in the cerebellum and blood were regarded as standards ($= 1$) and the specific activities measured in the other organs were referred to them (BORELL and ÖRSTRÖM, 1945—1947, BORELL, WESTMAN and ÖRSTRÖM, 1947). The determination of the specific activity alone has the advantage that the factor of correction is reduced and directer values of the phosphate metabolism are thus obtained.

If the concentrations of the radioactivity between the animals were always equal and the biological variations negligible the specific activity would be adequate and yield comparable values. The concentrations of P^{32} , however, vary more or less not only between the animals but also between different series of experiments. Hence, one has to refer the specific activity to a standard ($=$ relative specific activity), otherwise the values are not comparable.

In earlier papers (BORELL, ÖRSTRÖM, 1945—1947, BORELL, WESTMAN, ÖRSTRÖM, 1947) we have used the specific activity of the cerebellum as a standard in the determination of the phosphate turnover in the different parts of the brain. Naturally, also blood can be used as a standard. Many authors, mainly HEVESY (1940), hold that the specific activity in the blood plasma would be best suited for use as a standard.

We used whole blood instead of blood plasma in our measurements of the relative specific activity, owing to the technical difficulties which the determinations on such small quantities of blood, as are accessible in experiments on rats, present.

In order to determine which specific activity, i. e. that in the cerebellum or that in the blood, is closer correlated to the organs studied, we made a series of determinations of correlation. Table 1 shows the results obtained.

The lower the value the lesser the degree of correlation. The statistical significance of the correlations was determined by the t -analysis.

Table 1.
The coefficients of correlation of the specific activity.

	r	t	degrees of freedom	p
Blood-cerebellum	0.676	5.19	32	< 0.001
» tuber cinereum	0.486	3.14	32	< 0.01
» adeno-hypophysis	0.498	3.15	30	< 0.01
» ovary	0.658	4.94	32	< 0.001
Cerebellum-tuber cinereum	0.764	6.69	32	< 0.001
» adeno-hypophysis	0.691	5.24	30	< 0.001
» ovary	0.703	5.60	32	< 0.001

Table 2.

The specific activity and the relative specific activity in the organs examined during different phases of the sexual cycle.

a. specific activity. b. relative specific activity (specific activity of cerebellum = 1) c. relative specific activity (specific activity of blood = 1).

Number of observations	Prooestrus			Oestrus			Metoestrus			Dioestrus		
	6			12			7			9		
	a	b	c	a	b	c	a	b	c	a	b	c
Tuber cinereum..	0.65	3.15	0.052	0.51	2.46	0.035	0.61	2.61	0.034	0.56	1.95	0.035
Adeno-hypophysis	3.33	16.4	0.28	2.92	14.0	0.20	3.43	14.5	0.31	3.11	12.6	0.21
Ovary	7.55	37.1	0.60	6.95	34.1	0.46	7.25	31.5	0.47	9.31	36.6	0.60
Cerebellum	0.21	1.0	0.018	0.21	1.0	0.014	0.24	1.0	0.014	0.26	1.0	0.017
Blood	12.7	62.6	1.0	15.1	75.8	1.0	16.0	69.6	1.0	15.9	70.6	1.0

From Table 1 it is evident that the degree of correlation of the tuber cinereum and adenohypophysis with the cerebellum is higher than with the blood. Irrespective of whether the cerebellum or the blood are used as standards the coefficients of correlation are statistically significant. Concerning the ovary the correlation with the blood is slightly higher than its correlation with the cerebellum. In either case the values obtained are statistically significant with p -values < 0.001 . Of all organs studied, the correlation of the cerebellum with the blood is highest.

The objection may be raised that the values used in the measurements discussed above are heterogeneous because possible variations caused by the cyclic phases have not been taken into account. However, the difference between the coefficient of correlation for each phase of the cycle and that which is found if all cyclic phases are taken together, is negligible.

It is thus evident that the cerebellum is best suited for use as a standard in the measurements of the relative specific activity in the tuber cinereum and adenohypophysis because the coefficient of correlation of the cerebellum is higher than that of the blood. As regards the ovary, both the cerebellum and the blood can be used as standards because here the coefficients of correlation are practically identical.

Results.

Table 2 and Fig. 1 show the results obtained.

Tuber cinereum and adenohypophysis. The tuber cinereum and the adenohypophysis have a very high phosphate turnover during prooestrus independent of how the values are computed. The phosphate turnover in the tuber cinereum reaches its peak during this phase of the cycle. During oestrus the values decrease. This

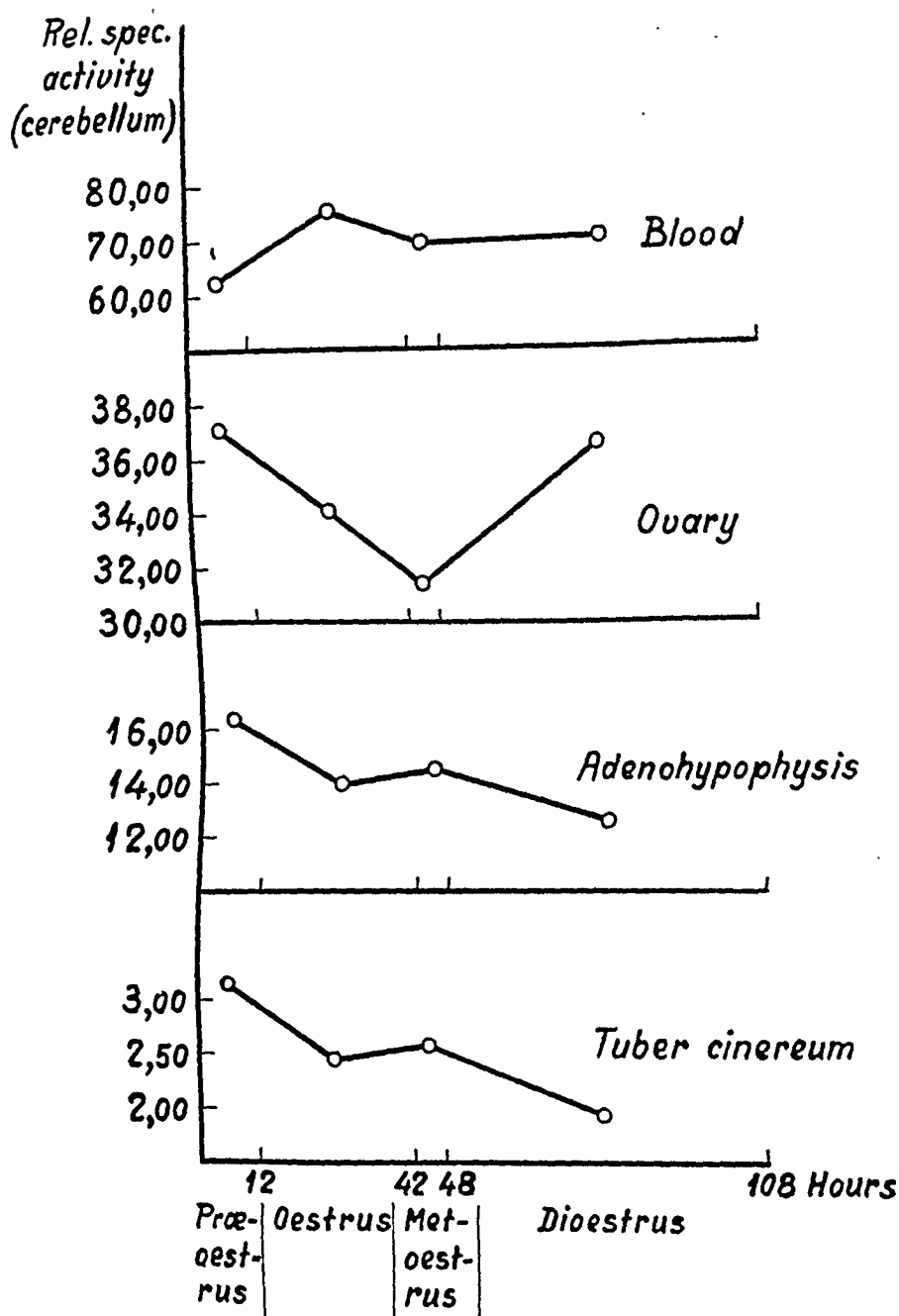


Fig. 1. The relative specific activity in the tuber cinereum, adeno-hypophysis, ovary and blood during different phases of the sexual cycle.

decrease is also noticeable if the relative specific activity is determined and blood used as a standard. After another rise during metoestrus the values fell again during dioestrus.

Table 3.

The P values of the difference between the phosphate turnover during the different phases of the sexual cycle.

Specific activity			Proestrus	Metoeestrus	Dioestrus
			< 0.3	— ¹	—
	Tuber cinereum	Oestrus	< 0.5	—	—
		Dioestrus	—	—	—
	Adeno-hypophysis	Oestrus	—	< 0.3	—
		Dioestrus	—	—	—
Relative specific activity (cerebellum = 1)	Tuber cinereum	Oestrus	< 0.05	—	—
		Dioestrus	< 0.001	< 0.05	—
	Adeno-hypophysis	Oestrus	< 0.2	—	< 0.4
		Dioestrus	< 0.2	—	—

Ovary. In the ovaries the specific activity is markedly higher during proestrus and dioestrus than during the other phases. This is particularly conspicuous if blood is used as a standard.

In Table 2 we have recorded also the values of the specific activity determined in the cerebellum and blood because here the variations do not run parallel with those observed in the other organs studied. Hence, the variations in the tuber cinereum, adenohypophysis and ovary are not brought about by generalized changes in the phosphate turnover.

The statistical significance of the results of the measurements discussed above was determined by the t-analysis. Values of $P \leq 0.01$ were looked upon as statistically significant and values between 0.01 and 0.05 were assessed as statistically probable. The results of these measurements show that the increase in the phosphate turnover in the tuber cinereum between dioestrus and proestrus is statistically significant and its decrease in this part of the brain between proestrus and oestrus, and between metoeestrus and dioestrus are statistically probable (Table 3).

From the statistical point of view, no differences in the phosphate turnover in the adenohypophysis were observed during the different phases of the reproductive cycle. It is, however, worthy of emphasis that these variations are similar in type to those observed in the tuber cinereum. In the ovary both the decrease in the phosphate turnover between proestrus and oestrus, and its increase during dioestrus are statistically significant.

¹ Means that the differences are obviously so small, that they can not be statistically significant.

In order to illustrate the importance of the measurements of the relative specific activity, we have recorded in Table 3 also some values of the specific activity in the tuber cinereum and adenohipophysis. These values show that measurements based on the specific activity do not yield any statistically significant differences. This is probably due to the great dispersion of the individual values. The relative specific activities, however, yield statistically probable and significant differences.

The difference in the specific activity in the tuber cinereum between dioestrus and prooestrus, for instance, has a p -value <0.5 whereas the corresponding difference in the relative specific activity has a p -value <0.001 .

Discussion.

The observations discussed in this study have shown that in rats the values of the relative specific activity in the tuber cinereum and adenohipophysis are high during prooestrus, *i. e.*, at such time as the development of the large follicles takes place. It is reasonable to assume that the intensification of the metabolic activity in the organs stated above is brought about by an increase in the activity in the diencephalon which in its turn causes an increase in the production of gonadotropic hormone. During oestrus the relative specific activity declines. There is a striking parallelism between these findings and our earlier observations on rabbits, *i. e.*, the phosphate turnover in the tuber cinereum and adenohipophysis begins to increase immediately after coitus and continues steadily to increase for 30 minutes. Then it decreases again with resultant low values at the time of ovulation. These findings suggest that the period of proestrus in spontaneously ovulating animals corresponds to the period of time just after coitus in animals in which mating induces ovulation.

The phosphate turnover in the *tuber cinereum* and *adenohipophysis* increases slightly also in rats during metoestrus, *i. e.*, at such time as the development of the corpus luteum takes place. The production of gonadotropic hormone is probably intensified also in this phase. During dioestrus the hormonal activity is obviously lesser than during the other phases.

The observation is very interesting that the phosphate turnover in the tuber cinereum runs parallel to that in the adenohipophysis during the different phases of the reproductive cycle. This obser-

vation lends further support to the assumption that the hypophyseal and diencephalic systems should be regarded as a functional unity.

In the *ovary* the phosphate turnover is high during prooestrus, i. e. when follicular maturation takes place. Another peak is observed during dioestrus. The variations in the phosphate turnover show that the ovarian metabolic activity is modified during each phase of the reproductive cycle. It is at present not possible to determine the functional importance of the changes in the phosphate turnover because experiments on pregnant and pseudo-pregnant animals have not yet been made. This problem will be dealt with in another paper.

Summary.

In the present study experiments were made on rats to demonstrate by means of radioactive phosphorus the phosphate turnover in the tuber cinereum, adenohypophysis and ovaries during the phases of the reproductive cycle. The following results were obtained.

- 1^o *Tuber cinereum*. The phosphate turnover reaches its peak during prooestrus. During the following phases it is markedly depressed.
- 2^o *Adenohypophysis*. The phosphate turnover is considerably greater than in the tuber cinereum. Also in the adenohypophysis maximum metabolic activity was observed during prooestrus.
- 3^o *Ovary*. The phosphate turnover is most intense during prooestrus and dioestrus.
- 4^o These observations lend further support to the assumption that, from the functional point of view, the hypophyseal and diencephalic systems should be looked upon as a unity.

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On the Possible Significance of the Lymphoid Organs for the Production of Serum Proteins in the Rat.¹

By

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Conflicting views prevail in the literature about the site of formation of serum proteins. Concerning fibrinogen and albumin evidence is accumulating to indicate that these proteins come from the liver, but in the debate about the source of globulin hardly an organ or body cell escapes attention as the possible site of formation.

For several years we have been interested in the function of the lymphocytes and plasma cells. According to ANDREASEN (1939, 1943, 1946) the small lymphocytes are "protein cells" of significance in the normal protein metabolism whereas studies of BING and co-workers (1937, 1940, 1944) show that there are reasons for assuming that the plasma cells play a part in the formation of serum globulin. Recent contributions from other laboratories on lymphocytes (DOUGHERTY, CHASE and WHITE 1944, WHITE and DOUGHERTY 1945, 1946 and EHRICH 1946) and on plasma cells (BJØRNEBOE and GORMSEN 1943, BJØRNEBOE, GORMSEN and LUNDQUIST 1947 and FAGRÆUS 1946, 1948) point in the same direction and indicate that studies on the formation of serum globulin now center about these two types of cells. In FREUND's review the literature, up to 1947, is summarized.

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Both lymphocytes and plasma cells are characteristic components of the lymphoid organs; it was natural therefore that we in the course of working out an experimental technique for the subtotal removal of the lymphoid organs tried to get additional information about the influence of these organs on the serum proteins.

Material and Method.

The animals employed were 98 inbred rats of ages between $2\frac{1}{2}$ and $8\frac{1}{2}$ months. The operative method has been described in this journal (ANDREASEN and GOTTLIEB, 1947). In most cases the present investigations comprised subtotal extirpations, i. e. combinations of thymectomy, splenectomy and subtotal adenectomy (25 animals). The first 5 subtotal extirpations were made in three seances, thymectomy first, the neck operations about two weeks later, and laparotomy after a further three weeks. The rest of the animals was operated in one seance. In this series the removal of the thoracic lymph nodes was not so radical as it is technically possible, only the most easily accessible of the bronchial nodes being removed. For comparison we performed isolated thymectomy in a few cases (4 animals) and in others splenectomy plus subtotal adenectomy, preserving the thymus and all bronchial lymph nodes (4 animals).

The control material was twofold: "Negative" control with 37 normal animals, and "positive" control of 28 sham operated animals. The latter were treated in a manner which, in respect of anesthesia, incisions, bleeding, suturing and other handling was as close an imitation of the actual operations as possible when the lymphoid organs in the field of the operations were to be left intact.

For the protein determinations we used heart blood taken in direct association with the killing of the animals. After opening the thorax under ether anesthesia the blood was caught in dry centrifuge tubes from a snip in the heart wall itself. In 3 cases while taking the blood samples we observed an admixture from the thorax of a turbid, milky fluid of unknown origin. These samples gave analysis results deviating greatly from the others and have therefore been left out of consideration.

The serum protein determinations were made by HENRIQUES and CLAUSEN's method with KJELDAHL determination and fractionating the proteins by means of semisaturation with ammonium sulphate at a constant pH and constant total protein content.

Electrophoresis experiments have been conducted under the following conditions: Undiluted sera were dialyzed for 48 hrs. at 5° C. against buffer (sodium phosphate, ionic strength 0.1, sodium fluoride, ionic strength 0.1, pH 7.7). After dialysation sera were diluted with buffer to about 1 per cent. content of protein (Δn 0.00183); the sera

in experiment no. 5 and 12 however were diluted still more. The potential gradient was about 6 volts/cm.; the temperature in the thermostat about 0.5° C. The experimental period varied from 17 400 to 21 420 sec.

Serum Proteins in Normal and Operated Rats.

Normal Rats. The results of the protein analyses on serum from 37 rats are presented graphically in fig. 1, where they are grouped according to the age and sex of the animals. Owing to the small number of animals used and their uneven distribution according to the age and sex, we cannot decide whether there is any variation due to these factors, except that a group of young females (4½ months old) seems to be placed at a somewhat higher value in comparison to a group of older males (8½ months old); the average values of total serum proteins in females and in males are 5.85 and 5.41 per cent. respectively.

The difference seems to affect the albumin as well as the globulin, the average values in female and male rats being 3.39 and 3.15 per cent. albumin and 2.50 and 2.24 per cent globulin respectively. The relative albumin percentage, *i. e.* the percentage of albumin to the total protein, varies between 50 and 68 per cent. throughout this normal material, with the same average value: 58 for both sexes.

Sham operated control animals. The results of the analyses are shown in the table 1. The total serum protein was found to be unchanged, whereas there in some cases was a transitory increase of the globulin values, which in a few cases rose to over 3.00 per cent. At the same time the albumin percentage was correspondingly lower, so that the total serum protein was maintained. Accordingly, the relative albumin percentage was found to be reduced (the lowest value found being 44 per cent.).

The changes may be due to inevitable slight infections. Post-operative wound processes therefore cause formation of antibodies.

Operated animals. The results of the analyses are given in table 2 and are presented graphically in fig. 1 together with the values from the normal rats. No change was found in the total serum protein. The globulin values varied in some cases during the first part of the post-operative period in the same manner as in some of the sham operated animals. For example in rat no. 231,

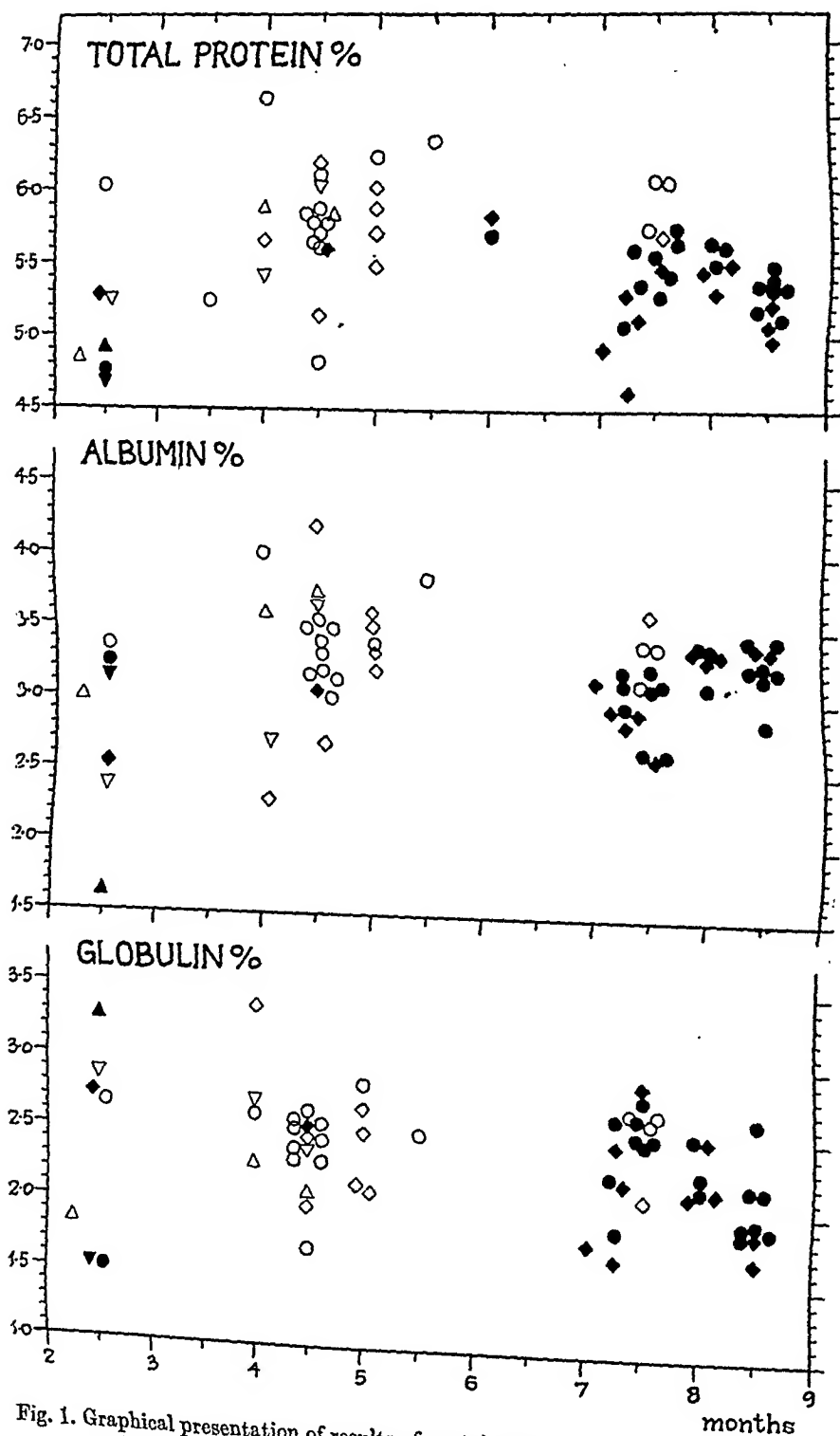


Fig. 1. Graphical presentation of results of protein analysis on serum from normal rats and after different sorts of extirpations of lymphoid organs.

Females males

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▲

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Normal animals,

After subtotal extirpations,

After adenectomy plus splenectomy,

After thymectomy.

Table 1.
*Results of Protein Analysis on Serum from Sham
 Operated Rats.*

No.	Age	Weight	Sex	Days after operat.	Total Protein	Albumin	Globulin	Relative Albumin
After sham subtotal extirpation								
	months	g.			%	%	%	%
745	2 1/2	163	♂	2	5.51	2.93	2.58	53
247	2 1/2	118	♀	2	5.10	3.21	1.89	63
739	2 1/2	153	♀	2	5.49	2.90	2.59	53
749	2 1/2	155	♀	2	5.74	2.96	2.78	51
736	2 1/2	155	♀	3	5.60	3.00	2.60	54
254	2 1/2	137	♂	7	5.13	2.36	2.77	46
234	4	125	♀	10	5.60	2.60	3.00	48
803	7 1/2	300	♂	14	5.48	2.61	2.87	47.6
802	7	247	♂	15	5.53	2.85	2.68	51.5
801	7 1/4	266	♂	25	5.03	3.07	1.96	61.1
796	7 1/4	274	♂	30	5.27	3.04	2.23	57.6
791	7 1/4	234	♂	33	5.26	2.98	2.28	56.6
236	4 1/2	158	♀	34	6.28	3.73	2.55	60
263	5	191	♀	34	6.20	3.14	3.06	51
259	5	170	♀	44	6.28	3.64	2.64	58
260	5	177	♀	45	6.10	3.06	3.04	50
257	5	160	♀	46	5.98	3.65	2.33	61
788	8	226	♂	57	6.30	3.56	2.74	56.5
786	8	216	♂	61	5.52	3.47	2.05	62.8
782	8	210	♂	65	5.68	3.24	2.44	57
After sham thymectomy								
243	2 1/2	129	♂	2	4.90	3.39	1.51	69
250	2 1/2	119	♀	7	5.30	2.38	2.92	45
233	4	181	♀	10	5.39	2.88	2.51	54
225	4 1/2	194	♀	35	6.03	3.67	2.36	61
After sham adenectomy and splenectomy								
245	2 1/2	112	♀	2	5.03	3.18	1.85	63
252	2 1/2	134	♀	7	5.28	2.31	2.97	44
230	4	142	♀	10	5.76	3.35	2.41	58
227	4 1/2	184	♀	35	5.85	3.72	2.13	64
Average of all sham operated rats					5.59	3.10	2.49	55.6

we found on the 10th day 3.36 per cent. globulin, 2.29 per cent. albumin and a relative albumin percentage of 41. No difference was found in the results of the different operations.

The electrophoresis studies were carried out with blood from all three groups of rats. Blood samples were examined at different intervals (varying from 15 to 64 days) after subtotal extirpations

Table 2.

Results of Protein Analysis on Serum from Operated Rats.

No.	Age	Weight	Sex	Days after operat.	Total Protein	Albumin	Globulin	Relative Albumin
After subtotal extirpation								
	months	g.			%	%	%	%
738	4 1/2	174	+	2	5.14	2.68	2.46	52
734	4 1/2	148	+	3	5.59	3.05	2.54	55
253	2 1/2	122	+	7	5.28	2.54	2.74	48
231	4	151	+	10	5.65	2.29	3.36	41
805	7 1/2	288	+	14	5.47	2.60	2.87	47.5
799	7	205	+	15	4.92	3.15	1.77	64.1
800	7 1/4	218	+	28	4.62	2.95	1.67	63.8
795	7 1/4	220	+	30	5.29	2.84	2.45	53.9
794	7 1/4	171	+	34	5.12	2.93	2.19	57.1
239	4 1/2	174	+	34	6.18	4.20	1.98	68
262	5	184	+	34	5.87	3.20	2.67	55
261	5	182	+	44	5.70	3.61	2.09	63
258	5	166	+	45	5.47	3.33	2.14	61
256	5	175	+	46	6.01	3.51	2.50	58
38	6	208	+	48	5.82			
792	8	201	+	59	5.30	3.30	2.50	56.9
789	8	190	+	61	5.50	3.36	2.14	61
785	8	208	+	64	5.45	3.34	2.11	61.2
46	8 1/2	258	+	c. 120	5.07	3.39	1.68	67
44	8 1/2	215	+	c. 120	4.97			
40	8 1/2	220	+	c. 120	5.22	3.36	1.86	64
78	7 1/2		+	c. 165	5.69	3.61	2.08	64
After thymectomy								
241	2 1/2	141	+	2	4.68	3.14	1.54	67
249	2 1/2	115	+	7	5.25	2.38	2.87	45
238	4	161	+	10	5.41	2.70	2.71	50
224	4 1/2	190	+	35	6.02	3.65	2.37	61
After adenectomy and splenectomy								
244	2 1/4	120	+	2	4.85	3.00	1.85	62
251	2 1/2	125	+	7	4.92	1.64	3.28	33
235	4	164	+	10	5.88	3.60	2.28	61
226	4 1/2	174	+	35	5.83	3.75	2.08	64
Average of all operated rats					5.41	3.11	2.31	57.3

of the lymphoid organs, the operations being performed in one séance. No significant difference between the groups is demonstrated (table 3). The relative percentage of albumin in the table contains a fraction of protein, amounting from 2.6 to 5.5 per cent. of the total serum protein. In two cases (no. 5 and 10) this fraction was not

Table 3.

Results of Electrophoresis Experiments on Serum from Normal Rats, after Sham Subtotal and after Subtotal Extirpation.

No.	Mixture of sera		Total Protein		Relative Albumin	Relative Globulin				
	Vol.	Rat No.	Undil.	After Dil.		α		β		γ
						α_1	α_2	β_1	β_2	
Normal rats										
	ml.		%	%	%					
6	{2.4	790	5.15	1.11	65.6	5.4	5.2	10.8	2.9	10.2
	{0.7	793								
8	{1.5	804	5.35	1.11	61.4		11.7	10.9	4.5	11.7
	{1.9	806								
	{0.7	783								
10	{0.8	787	5.60	1.08	61.3		9.9		11.8	17.0
	{0.7	798								
Average			5.37		62.8		10.7		13.6	13.0
After sham subtotal extirpation										
3	{1.5	236	6.21	1.09	56.7	8.7	3.9	12.2	5.8	12.6
	{0.5	257								
4	{1.4	260	6.15	1.09	55.8		12.7		19.6	11.9
	{1.5	263								
7	{1.2	791	5.27	1.09	63.4		11.7		13.4	11.6
	{1.5	796								
	{0.9	782								
11	{0.9	786	5.80	1.13	67.3		11.2	7.9	4.6	9.1
	{0.8	788								
Average			5.86		60.8		12.0		15.9	11.3
After subtotal extirpation										
1	{0.6	258	5.62	0.98	63.5		11.5		13.1	12.0
	{1.4	261								
2	{1.6	256	5.94	1.16	57.8		11.7	11.1	3.1	16.3
	{1.8	262								
5	{1.1	799	4.80	0.81	64.9		11.5		12.6	11.0
	{0.8	800								
	{1.1	785								
12	{0.6	789	5.50	0.60	62.0		14.4		10.8	12.9
	{0.5	792								
Average			5.47		62.0		12.3		12.7	13.0

observed, but otherwise it was found walking in front of the albumin on the ascending side and in 5 cases in addition on the descending side with a mobility of about $5.50 \cdot 10^{-5}$ cm²/volt sec.

Discussion.

Studies by WHIPPLE (1942, 1944), SCHOENHEIMER, RATNER, RITTENBERG and HEIDELBERGER (1942 a) and others have shown that the plasma proteins are in a steady state of dynamic interchange with the amino acids and proteins of the tissues. The antibody proteins are involved in just the same cycle of metabolic reactions as the other proteins of the blood plasma. By following the rate of replacement of the nitrogen isotope by normal nitrogen it was demonstrated (SCHOENHEIMER et al., 1942 b) that the half life of an antibody molecule is about 2 weeks, approximately the same as that of the average serum proteins. In other words, the plasma proteins are not stable compounds with a low rate of "regeneration" as previously supposed. Consequently, the removal of the organs or tissues supposed to be responsible for the production of a plasma protein may soon after influence the normal amount of the circulating protein in question.

To which extent we succeeded in extirpating the lymphatic organs has been estimated on the basis of our present knowledge of the quantitative anatomy of these organs in the rat at different ages (ANDREASEN 1943). After the subtotal extirpations only some few lymph nodes (bronchial and popliteal) as well as the PEYER's patches are left. These remnants make a total of about 10 per cent. of the values found in normal animals. *By the subtotal extirpations about 90 per cent. of the organoid lymphoid tissues are removed.* Performing subtotal adenectomy plus splenectomy or isolated thymectomy we extirpated about 60 per cent. and 30 per cent. of the organoid lymphoid tissue respectively.

In these experiments we have demonstrated that even extensive extirpations of the lymphoid organs in rats do not influence significantly the normal range of serum proteins. This would seem to dispose of the lymphoid organs as an essential factor in serum protein production in this animal.

An obvious objection to this argument would be that the operations performed were not radical enough. Hyperplasia of the remnants of the lymphoid organs, resulting in re-establishment of their function, would rapidly compensate for any deficiency in serum proteins. However, at autopsy we found no or only slight quantitative changes of the few small lymph nodes

left, and certainly nothing like a re-establishment of the normal quantity. Obviously, spleen and thymus never regenerate. Furthermore, the demonstrated lymphopenia induced by the operations (ANDREASEN and GOTTLIEB, 1947) suggests a profound, but transitory, interference with the functions of the lymphoid organs.

The fact that the normal level of serum proteins can be maintained in spite of almost complete absence of the lymphoid organs indicates the probability of serum protein production occurring somewhere outside the lymphatic organs. However this does by no means exclude a conception of the lymphoid cells as the source of serum proteins. A large number of these cells exist as non organoid components of the connective tissues and probably they are able to continue the functions of the organoid parts of the lymphoid system which were removed in the operated animals, *e. g.* the mucosa of the intestine contains plenty of plasma cells which may be active elements in the formation of globulin. It is possible that histological quantitative studies of the connective tissues of the operated animals may contribute to the elucidation of this particular problem.

Summary.

By means of subtotal extirpation (comprising until 90 per cent.) of the lymphoid organs in the rat an attempt has been made to throw some light upon the possible connexion between these organs and the formation of serum proteins. In the early period after the operations an unspecific, slight increase of the serum globulin and a corresponding decrease of the serum albumin was demonstrated. After cessation of the mesenchymal reactions due to wound healing the serum proteins were found within the normal range. This seems to dispose of the organoid lymphoid tissue (thymus, lymph nodes and spleen) as an essential factor in the production of serum proteins.

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Observations on Reactive Hyperaemia as Related to Histamine, on Drugs Antagonizing Vasodilatation Induced by Histamine and on Vasodilator Properties of Adenosinetriphosphate.

By

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The idea that the dilatation of the small blood vessels in tissues rendered ischaemic by obstruction of the main blood supply, develops as a result of the liberation and accumulation in the ischaemic tissues of vasodilator substances has been mooted by many investigators. LEWIS (1927), in extensive studies on the human skin, concluded that reactive hyperaemia is due to the liberation in the tissue spaces of H-substance, a normal vasodilator metabolite, and that this substance is histamine, free or held in loose combination.

Attempts to demonstrate, by a direct approach, the liberation of histamine during reactive hyperaemia have given inconsistent information. The literature has recently been reviewed by EMMELIN et al. (1941) and by KWIATKOWSKI (1941). In experiments in this laboratory, during reactive hyperaemia in the dog's ear, the dog's forelimb and the human arm, no increase in the histamine concentration of the venous plasma or blood emerging from the ischaemic tissues could be demonstrated by the BARSOU-GADDUM method; in guinea-pigs and cats, using the bronchiolar tone, the gastric secretion and the arterial blood pressure as indicators, we found no evidence of excess histamine in the blood, when the

abdominal aorta was unclamped after having been obstructed for 10 to 20 minutes underneath the renal arteries (EMMELIN, KAHLSON and WICKSELL 1941). Our observations are in agreement with those of KWIATKOWSKI.

BILLINGS and MAEGREATH (1938) on the other hand, working on the hind limbs of rabbits, arrived at positive results similar to those originally reported by BARSOUM and GADDUM (1935) and claim that, in addition to histamine, a substance with the biological properties of adenosine can be detected in the ischaemic tissues and the venous blood returning from the limb.

LEWIS (1927) concluded from observations on the human skin that any substance that could be responsible for reactive hyperaemia does not easily diffuse into the vessels. If this is so, excess histamine in detectable quantities is not likely to appear in the venous outflow from ischaemic regions and a new mode of approach seems necessary to obtain relevant evidence. In the experiments to be described here a) we have, in a series of cats and dogs with wide individual variations in the vasodilator response of the hind limbs to injected histamine, studied quantitatively the relations between sensitivity to histamine and the magnitude of reactive hyperaemia in the hind limbs; b) we have investigated if, and to what extent reactive hyperaemia is interfered with when the small blood vessels have been rendered completely insensitive to the vasodilator action of histamine in physiological amounts; c) incidentally we made some observations on the failure of naturally accumulated metabolites to dilate constricted vessels, where histamine and other stimuli were effective in producing vasodilatation; d) finally we made some observations on the vasodilator properties of adenosinetriphosphate.

Methods.

Our experiments were performed on cats and dogs under chloralose, initially anesthetized with ether. The animals were eviscerated, leaving the kidneys and the liver with its arterial supply intact. The abdominal aorta and the iliac arteries were freed and all branches not supplying the hind legs tied. The vena cava inferior was exposed and all subsidiaries draining tissues other than the legs were ligated. At the level of the fifth lumbar vertebra the body wall was mass-ligated with strong twine in three sections. A tightened plastic band was arranged around the trunk to obstruct small collateral channels, avoiding interference with the sympathetic chains. Heparin was injected into a vein in doses sufficient to prevent clotting for a couple of hours; when

necessary additional heparin was injected. For injection of drugs a cannula was inserted into the central stump of the inferior mesenteric artery. Arterial blood pressure was recorded from the brachial artery using a mercury manometer.

For measuring the rate of blood flow through the leg a Gaddum (1929) outflow recorder was mounted on the venous side, a collecting

cannula being inserted in the distal stump of the vena cava, from where the blood through the recorder collected in a funnel which served as a venous reservoir; from there it was returned to the heart through a cannula inserted in the proximal part of the vena cava. To prevent undue rise of capillary pressure in the legs these were raised so that the knees were at the same level as the recorder.

Reactive hyperaemia (R.H.) was produced by obstructing the aorta; concomitantly the venous return from the reservoir was discontinued by tightening a clamp. With this technique there was no perceptible collateral circulation to the hind limbs, since during obstruction of the arterial inflow, the venous out-

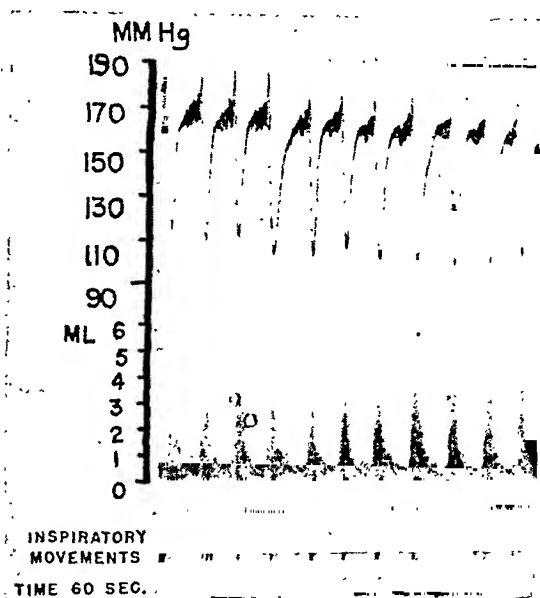


Fig. 1. Dog, 6 kg under chloralose. Reading from below: Time marks 60", inspiratory movements, signal, rate of blood flow through the hind limbs, pressure in the brachial artery. Spontaneous attack of Cheyne-Stoke's breathing. During the periods of apnoe and accumulation of CO_2 the blood flow in the limbs is progressively reduced as a result of vasoconstriction; concomitantly the blood pressure in the brachial artery rises.

flow from the legs was nil, and blood even passed backwards into the legs, obviously due to vasodilatation in the ischaemic tissues.

To avoid draining blood from the animal, the venous reservoir at the beginning of the experiment, was filled with either heparinized blood from another animal or with an 8 % solution in Tyrode of "Dextran", a polymerized sugar of high molecular weight (GRÖNVALL and INGELMAN 1944).

In our experiments the rate of flow through the legs and the blood pressure remain approximately constant during a couple of hours. At a later stage blood flow through the limbs decreases progressively and may, after several hours of perfusion, be reduced to nil. There is the possibility of an insignificant collateral drainage by vessels supplying the spinal channel which in controls performed in the course of another series of experiments amounted to less than 1 to 2 ml per minute.

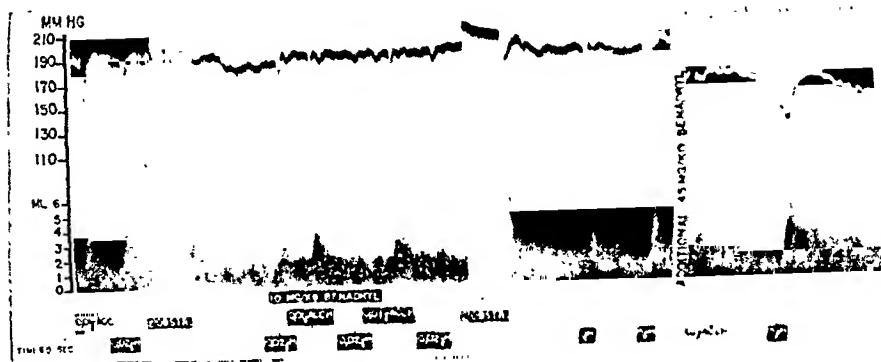


Fig. 2. Cat, 3 kg under chloralose. Time marks in 60", signals indicating injections and obstruction, signal indicating infusion of histamine antagonist, rate of blood flow in the hind limbs, pressure in the brachial artery.

During the first 1—2 hours of the experiment the blood vessels and the vasomotor control are in a satisfactory state as demonstrated in an experiment part of which is shown in fig. 1.

Fundamentally similar procedures as those described above will be used in a series of experiments to be published later from this laboratory. Incidentally we stress the well known fact that recording the arterial blood pressure alone is a very poor and fallacious indicator of vasodilator responses. To exemplify this, we give the following figures: in the experiment referred to in fig. 2, in the initial state of R.H. following 2 minutes of obstruction of blood supply, the fall in blood pressure amounts to about 70 mm Hg, whilst the corresponding increase in flow is about 100 %; in another experiment (fig. 4) the corresponding figures are 40 mm Hg and 500 %.

Results.

A. Quantitative Assay of R.H. and Vasodilation Produced by Histamine.

The rate of blood flow through the hind limbs was measured in 24 cats. Histamine diphosphate, dissolved in 0.2 ml warmed Tyrode's solution, was injected from the artery into the limbs. We satisfied ourselves that at the beginning of the actual experiment, injections of Tyrode's solution had no effect on the rate of flow. The dose of histamine necessary to produce an increase of 25 to 50 % in the rate of flow during the first 30 seconds subsequent to the injection, varied considerably with the individuals, the extreme figures being 0.003 γ /kg in the most sensitive cat and 0.24 γ /kg in the least sensitive; the average figure for the twelve most sensitive cats was 0.005 γ /kg and 0.05 γ /kg for the twelve

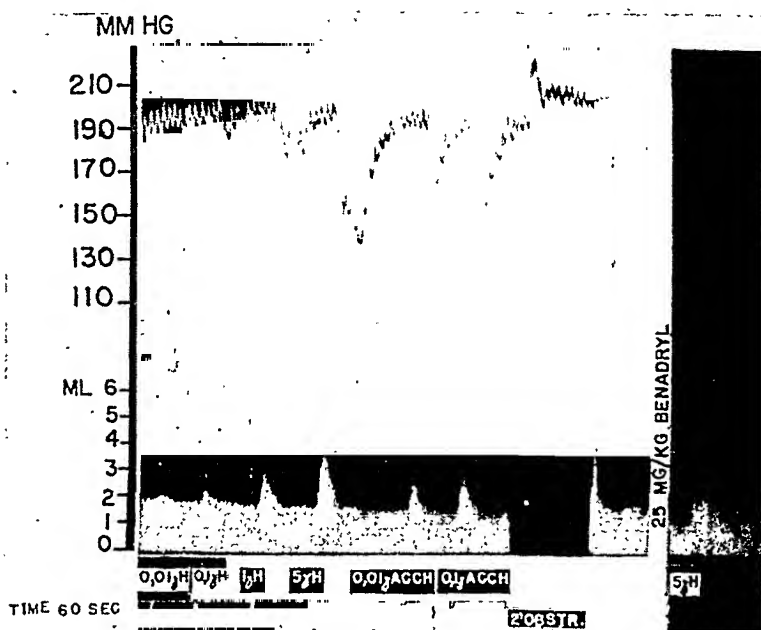


Fig. 3. Cat, 4.5 kg under chloralose. Signals and registrations as in fig. 2.

least sensitive. The distribution of sensitivity among the entire group is not uniform; there is a tendency to aggregate in two distinct groups around these two mean values.

Since in our experiments increase in rate of flow occurred simultaneously with a conspicuous fall in arterial pressure, increase in flow is taken to indicate dilatation of the vessels in the limbs, predominantly the arterioles. In every instance histamine in effective doses caused vasodilatation, constriction never occurred. Fig. 2 represents part of a tracing obtained from a cat of high sensitivity to histamine. Intraarterial injection of 0.02γ histamine causes a striking vasodilatation. Following this injection the blood flow in the limbs was obstructed for two minutes. During the subsequent R.H. the rate of flow is initially approximately doubled.

Fig. 3 is from a cat of extremely low sensitivity to histamine. 1γ histamine produces a smaller vasodilatation than 0.02γ in the cat referred to in fig. 2. The R.H. following two minutes of obstruction of blood flow, is however, initially approximately doubled as in the cat of fig. 2, the vessels of which are at least fifty times more sensitive to injected histamine.

Figures 2 and 3 incidentally demonstrate an observation made by us in many experiments; there is no correlation between the

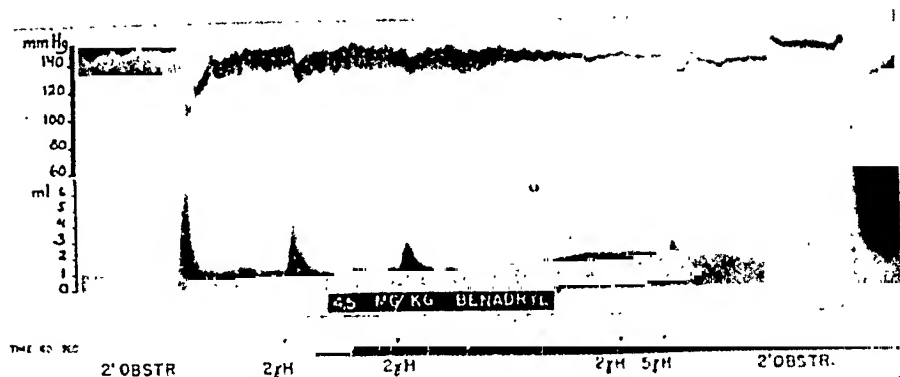


Fig. 4. Dog, 6.5 kg under chloralose.

sensitivity of the small vessels to the dilator effect of histamine and acetylcholine; the blood vessels may, as in fig. 3, be extremely insensitive to histamine, but highly responsive to acetylcholine.

In dogs, under the conditions described, the sensitivity of the blood vessels to histamine is of about the same order as in the group of cats of low sensitivity. Fig. 4 represents a typical experiment on a dog. R.H. following a two minute obstruction amounts initially to about six times the normal flow. In this series we made observations on six dogs.

From experiments of this type on cats and dogs it is obvious that the vasodilatation in R.H. is of the same magnitude in animals where the blood vessels are relatively insensitive to histamine as in animals of high sensitivity. Even in animals where the difference in sensitivity to histamine varies by a factor of 50 to 100, there is no conspicuous difference in the magnitude of vasodilatation during R.H.

B. R. H. after Rendering the Blood Vessels Insensitive to Histamine.

It has been shown by several workers that benadryl and related compounds counteract the depressor effect of histamine when judged by recording the arterial blood pressure. Experiments to study more quantitatively the anti-histamine properties of these drugs on the arterioli have, to our knowledge, so far not been devised.

The anti-histaminic drugs were infused slowly and continuously, during 10 to 30 minutes. During the course of the infusion, histamine was injected in increasing doses. This procedure made it

possible to evaluate exactly to what extent the sensitivity of the small blood vessels to histamine had been reduced below normal, and what quantities of antagonistic drugs had been necessary at any given moment. At suitable intervals during the course of decreasing vasodilator response to histamine R.H. was produced and quantitatively assayed. In the experiment on a cat, shown in fig. 2, benadryl, 10 mg per kg body weight, was infused in ten minutes. In this experiment 2.5 mg/kg benadryl conspicuously reduced the vasodilator effect of 0.02 γ histamine, 6 mg/kg almost entirely abolished this effect. After 10 mg/kg benadryl 1—5 γ histamine is required to produce a vasodilatation of the same magnitude as initially caused by 0.02 γ ; the sensitivity of the vessels to histamine is reduced to 1/50—1/250 of normal. The sensitivity of the vessels to histamine can not be reduced below this limit by a further supply of benadryl. The second part of the tracing (fig. 2) shows the situation after an additional infusion of 45 mg/kg benadryl. The vasodilator response to 5 γ histamine is of the same magnitude as after 10 mg/kg.

In all experiments of this type on cats, maximal antagonistic effect towards the vasodilator action of histamine was obtained by benadryl 6—10 mg/kg. Under the influence of this dose of benadryl, the whole population of cats investigated, no matter whether extremely sensitive or insensitive to histamine, was uniform in the vasodilator response: 1—5 γ histamine was required to produce a conspicuous vasodilatation. Fig. 3 demonstrates this fact on a cat of low sensitivity to histamine.

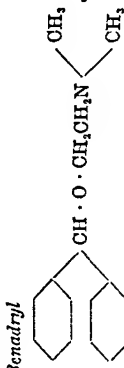
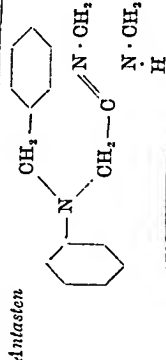
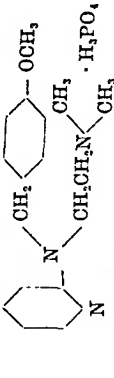


Fig. 2 demonstrates the magnitude of vasodilatation during R.H. in a phase of the experiment, when the sensitivity of the vessels was reduced to 1/50—1/250 of normal: at this stage the magnitude of R.H. is at least as big as under normal conditions.

Incidentally it may be mentioned that our experiments confirm the observation that benadryl antagonizes the vasodilator action of acetylcholine; this antagonism, however, is not so striking as that towards histamine, 10 mg/kg benadryl reducing the sensitivity to about 1/10 of normal.

Fig. 4 represents observations on a dog. Benadryl, 4.5 mg/kg, entirely abolishes the vasodilator action of 2 γ histamine. R.H. during the phase of reduced response of the blood vessels to histamine is of at least the same magnitude as normally.

Figs. 2 and 4 indicate that benadryl interferes in some way or other with the vasomotor regulation: in the normal state, during

Table 1.

Substance	Dose necessary to annul maximal vasodilatation caused by minimal dose of histamine	Lethal dose	Atropinelike effect on blood vessels
<i>Benadryl</i> 	6-10 mg/kg	45-60 mg/kg	Reduces sensitivity to acetylcholine to about 10 % of normal
<i>Antasten</i> 	2-3 mg/kg	30-40 mg/kg	Insignificant
<i>Neoantergan</i> 	0.6-1 mg/kg	20-30 mg/kg	None
<i>MMB¹</i> 	Rather toxic. Exerts a remarkable pressor activity. Anti-histamine effect is weak.		
<i>DMB²</i> 	Potent pressor substance: no anti-histamine and no atropine-like effect, of about equal toxicity as MMB.		

¹ and ² A product of H. Lundbeck & Co., Ltd., Copenhagen. We are indebted to this firm for generous supply of these drugs.

obstruction of the abdominal aorta, there is none, or only a transient rise in arterial blood pressure, whilst, under the influence of benadryl the reflex regulation is disorganized. In the experiment of fig. 4 the fluctuations in blood pressure, mainly related to the respiration, are abolished under the influence of benadryl.

Experiments of this type were also performed on cats and dogs using "neoantergan" or "antasten" for depressing the vasodilator response to histamine. The results with R.H. were fundamentally the same as with benadryl. A relatively small dose of these drugs reduced the vasodilator action of histamine to 1/50—1/250 of normal; increasing this dose even to a lethal level did not further depress the sensitivity. Neoantergan and antasten antagonize the vasodilator action of acetylcholine to a far smaller degree than does benadryl, actually in our experiments there was hardly any regular antagonism towards acetylcholine. Quantitative data of these antagonisms are summarized in table 1.

C. Vasodilator Reactions in Limbs with Arrested Blood Flow due to Vasoconstriction.

After a few or several hours of perfusion the blood flow through the limbs decreases progressively whilst the arterial pressure remains at a high level. The condition can be aggravated and develops faster if 10—20 % of the total blood volume is withdrawn. This decrease in the rate of flow is obviously due to vasoconstriction in the legs. If, in addition, the pressure in the veins of the leg is raised by elevating the venous reservoir, the blood flow through the legs stops altogether. In our experiments this state could be produced regularly. We elevated the venous pressure just sufficiently to reduce the blood flow to nil. When vasoconstriction was very advanced this could be achieved by elevation of the venous reservoir by 5—10 cm. If the preparation was kept under these conditions, the blood flow in the limbs remained obstructed. This ischaemic state seems very favourable for the accumulation of those metabolites alleged to be instrumental in vasodilatation during R.H.

The situation described is shown in fig. 5 obtained from a cat. Blood flow in the legs is nil, arterial pressure rather high. At the beginning of the second part of the tracing the circulation in the limb has been arrested for about 15 minutes. Intraarterial injection of histamine into the constricted vessels in minute doses produces vasodilatation as does acetylcholine. Obviously the arterioli in

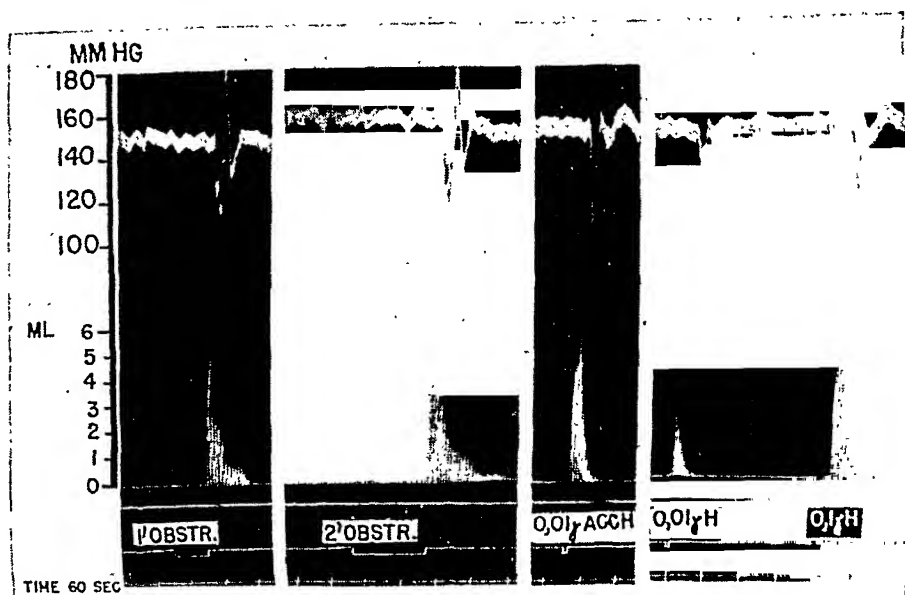


Fig. 5. Cat, 3 kg under chloralose. Cessation of blood flow in the limbs due to vasoconstriction and moderate elevation of venous pressure.

this state do respond to vasodilator substances, if they are present in active concentrations.

The first part of the tracing shows a remarkable phenomenon. If, in the state of advanced vasoconstriction with arrested blood flow, the aorta is obstructed for a short period, vasodilatation is elicited, obvious from a considerable outflow from the veins. We observed this effect even when obstructing the artery for as short a period as 5—10 seconds. The vasodilatation is prolonged and intensified by increasing the period of arterial obstruction. This phenomenon will be studied more closely in further experiments.

D. Vasodilator Action of Adenosinetriphosphate (ATP).

It has been suggested by several workers that adenosine and other phosphoric compounds may play a part in vasodilatation during R.H. and in the tetanized muscle. We tested the effect of a very pure preparation of ATP¹, injected as sodium salt, on the vessels of the leg in cats. From fig. 6 it will be seen that ATP is an extremely potent vasodilator: 0.2 γ causes a definite effect, 2 γ more than doubles the rate of flow. Neither benadryl nor atropin interferes with the vasodilator action of ATP.

¹ We are indebted to Boots Pure Drug Co. Ltd., Nottingham, England, for generous supply of ATP.

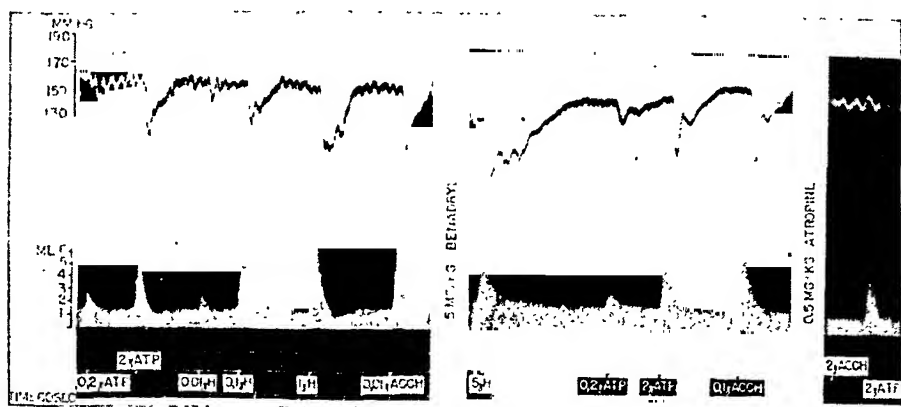


Fig. 6. Cat, 3.2 kg under chloralose. Action of adenosine triphosphate as compared with histamine and acetylcholine.

Comments.

In cats, under the conditions described, the sensitivity of the small blood vessels of the hind limbs to the vasodilator action of histamine varies individually within wide limits, some cats are 50 to 100 times more sensitive than others. Such differences might or might not be due to differences in details of the experimental procedures beyond our control. Whatever the reason, this situation offers a useful opportunity of comparing quantitatively the magnitude of reactive hyperaemia in these individuals. In our experiments the magnitude of reactive hyperaemia is completely independent of the sensitivity of the vessels to histamine. Even in cats, the small blood vessels of which are extremely insensitive to injected histamine, the magnitude and duration of reactive hyperaemia is of about the same order as in the most sensitive cats. In view of this fact it is difficult to conceive that vasodilatation during reactive hyperaemia should be due to a direct action of histamine on the small blood vessels. Again, it is difficult to assume that a blood vessel could be relatively insensitive to histamine reaching it from the artery but highly sensitive to histamine approaching it from the tissue spaces; further, it is hard to believe that there is no correlation in the sensitivity of the vessel to histamine reaching it by these two different routes.

The introduction of potent antagonists to histamine affords new possibilities in evaluating the rôle of histamine in reactive hyperaemia. In our experiments benadryl and related compounds completely annul the vasodilatation in the hind limbs produced by

intraarterial injection of histamine in quantities which might be considered physiological. Under the influence of these histamine-antagonists, in a state where the small vessels are completely insensitive to histamine in quantities which normally cause profound vasodilatation, the magnitude of reactive hyperaemia is not reduced. This is in agreement with EMMELIN and EMMELIN (1947) from this laboratory who observed that benadryl did not diminish the increase in volume of a cat's leg enclosed in a plethysmograph when the arterial inflow was re-established after a period of obstruction. Experiments of this type are open to the criticism that the antagonists employed might render the small vessels insensitive to histamine dispersed by the arterial route, but not to naturally liberated histamine accumulating in the surroundings of the small vessels. Such criticism is disproved by the fact, established by several workers, that benadryl and related compounds significantly reduce the vasodilatation produced by injection of histamine into the skin (literature reviewed by LOEW 1947).

Unfortunately the exact mode of action of these drugs in antagonizing the vasodilator effect of histamine is only poorly understood. WELLS et al. (1945) suggest a competition between histamine and benadryl for a given site of action or receptive substance and imply that if benadryl combines with the receptive substance it prevents histamine from combining on this same site; these authors further conclude that a constant per cent of any dose of histamine is antagonized by a given dose of benadryl. In our experiments the vasodilator action of histamine, in quantities 5 to 100 times the threshold dose, is not significantly reduced by doses of antagonists 10 to 25 times greater than the dose adequate to abolish completely the effect of a threshold dose of histamine; in the type of cat originally insensitive to histamine 1 mg/kg neoantergan may completely annul a profound vasodilatation caused by 0.5 γ histamine, whereas 25 mg/kg does not even reduce the vasodilatation produced by 5 γ histamine; this also holds for benadryl (fig. 2 and 3). This indicates that histamine given in a quantity exceeding the minimal dose required to produce maximal vasodilatation, combines with a receptive substance different from that for which benadryl and histamine in physiological doses are competing. In consequence histamine may cause vasodilatation by combining with two different types of receptors, one of which is not blocked to histamine even by lethal doses of histamine-antagonists. This assumption is supported by our observation

that under the influence of antihistaminic drugs the entire population of cats, with individual sensitivities to histamine varying in the range 1 : 100, is transformed to a population of nearly uniform sensitivity, the threshold dose causing profound vasodilatation now being 2 to 5 γ ; it seems to us reasonable to assume that this dose acts on a type of receptive substance which is not blocked by antihistaminic drugs.

In the experiments described in section C conditions seem very favourable for liberating and accumulating vasodilator metabolites. The small vessels in this state of profound constriction are very sensitive to dilating stimuli or agents, as shown by 1) unloading the arterioli from the pulsatile arterial pressure by occluding the artery elicits a vasodilator mechanism, possibly mechanical or nervous, revealed by the transitory reestablishment of blood flow on opening the artery (fig. 5); 2) histamine and acetylcholine injected from the artery in physiological concentrations produce vasodilation, where the naturally accumulated substances fail to do so. To us it seems inconceivable why histamine, if present in active concentrations in the tissue spaces, should be incapable of causing a vasodilatation which histamine reaching the arterioli by the arterial route easily produces. In addition, these experiments prove that histamine approaching the small vessels from the inside at least is not less active than histamine naturally accumulated outside the vessels. Observations recently published by EMMELIN (1945) are relevant at this point: he has shown that the blood vessels in the cat become insensitive to histamine injected in the artery if the vessels are exposed for some time to histamine in concentrations exceeding the normal plasma level.

Further experiments will be necessary to elucidate more directly and finally the rôle of histamine in reactive hyperaemia. To us the mechanism of reactive hyperaemia remains obscure.

Adenosinetriphosphate possesses potentialities as a natural vasodilator metabolite. GILLESPIE (1934), using the cat's blood pressure as indicator, observed a fall on injecting 0.1 mg adenosinetriphosphate in the jugular vein. FLEISCH and WEGER (1937), on perfusing a hind limb in cats and dogs, observed vasodilatation when the blood contained adenosinetriphosphate in the concentration 1 : 1,400,000. KALCKAR and LOWRY (1947) report, that the presence of 2 to 5 mg per cent of 3-adenylic acid or of about 1 mg per cent of 5-adenylic acid in the blood plasma was accompanied by a distinct lowering of blood pressure in the dog. The procedures

we employed, where only fractions of a γ were injected, seem to reveal adequately the powerful vasodilator properties of adenosinetriphosphate. In a following paper we hope to present a fuller account on this subject.

Summary.

1. A method is described for quantitative studies of reactive hyperaemia in the hind limbs of cats and dogs.

2. In cats the sensitivity of the small vessels to histamine varies within a very wide range, the magnitude of reactive hyperaemia, however, was found to be independent of the sensitivity of the vessels to histamine.

3. The magnitude of reactive hyperaemia is not reduced by rendering the small blood vessels completely insensitive to histamine in physiological concentrations.

4. Benadryl and related compounds completely annul the vasodilator effect of the minimal doses of histamine which produce maximal vasodilatation. The effect of larger doses of histamine, 5 times greater than the minimum dose and upwards, depending on the basic sensitivity to histamine, is not annulled or even diminished by any amount of antagonistic drug.

5. It is suggested that there are two types of receptors sensitive to histamine only one of which can be blocked by benadryl and related compounds.

6. It is demonstrated that adenosinetriphosphate is a very potent vasodilator, 0.1—0.2 γ causing profound vasodilatation in the hind limbs when injected in the artery.

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The Influence of g-Strophanthin on Hypodynamic and Anoxic Heart Muscle of the Frog.

By

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Received 26 January 1948.

In a previous paper one of us (LUNDIN 1946) investigated the influence of g-strophanthin on the mechanical properties of frog's cardiac muscle. These first experiments were performed on parallel bundled muscle preparations from the cardiac ventricle of the frog, working under aerobic conditions and considered normodynamic. In these experiments diastolic tension, extra tension during contraction, stiffness and viscosity at rest and during contraction remained unchanged after addition of g-strophanthin at various concentrations.

Investigations of CATTELL and GOLD (1938, 1940), SULZER (1932), BERG and BOER (1939) and others have shown that digitalis glycosides have a positive inotropic effect on hypodynamic myocardium from both warmblooded and coldblooded animals.

On the other hand KATZ et al. (1938), in experiments on the isolated heart of the dog, claim to have demonstrated that under physiological as well as pathological conditions digitalis glycosides have no effect on the force of contraction of the heart. The object of the present investigation is to supplement the previous reports already referred to with experiments on hypodynamic heart muscle preparations from the frog.

Method.

For further details of method we refer to previous papers (LUNDIN 1944, 1946). A preparation of parallel threaded muscle bundles from the cardiac ventricle of the frog, about 1.5 mm in length and 0.3 mm in thickness, was arranged in a chamber containing 5 ml of Ringer's solution (NaCl 6.70 g, $\text{CaCl}_2 + 6\text{H}_2\text{O}$ 0.4 g, KCl 0.2 g, glucose 0.2 g, dextran 30 g, distilled water to 1000 ml, NaHCO_3 added to pH 7.3). The chamber was closed by a tightly fitting glass cover in order to isolate the Ringer's solution from the atmosphere. A gas mixture containing various proportions of N_2 and O_2 and 1 % CO_2 was fed continuously through the Ringer's solution and allowed to escape through a hole in one of the corners of the cover. The O_2 content of the gas mixtures varied between 0.3 % and 70 %, determined with a Haldane-Krogh gas analysis apparatus. The pH of the solution in the chamber was constant as measured with a glass electrode. The temperature was kept either at 18° C. or at 4° C. Diastolic tension and extra tension during isometric contraction was recorded by means of a condensor myograph (BUCHTHAL 1942). Capacity variations of the condensor myograph, linear with the changes of muscle tension, were measured by a high frequency circuit amplifier and recorded with an electrostatic mirror-oscillograph or a galvanometer. The muscle bundle was stimulated at constant intervals, 8 per minute, with rectangular current impulses. The duration and the intensity of the impulse could be varied. Usually the duration was 5 milliseconds and the intensity slightly above threshold.

We performed 50 experiments each lasting for 1/2 to 4 hours. The muscle bundle in the chamber was stretched to slightly above equilibrium length and left to consolidate for 15 to 20 minutes before an experiment was started. Diastolic tension and extra tension during contraction were registered at 1–5 min. intervals. In some experiments excitability was also recorded. The excitability was determined from duration-intensity curves for the threshold stimulus between the ranges of 0.1 to 50 milliseconds.

G-strophanthin was added to the solution in the chamber to give concentrations varying between $5:10^7$ and $2:10^5$. A few experiments were made on muscle preparations which were treated for 20 min. with a solution of monoiodoacetic acid $2:10^5$ in Ringer's solution before g-strophanthin was added.

Results.

Hypodynamia, a condition in which the contraction power is reduced below the initial "normal" value, occurs spontaneously in a heart muscle preparation from a frog even when safeguarded in Ringer's solution with colloids and glucose (CLARK

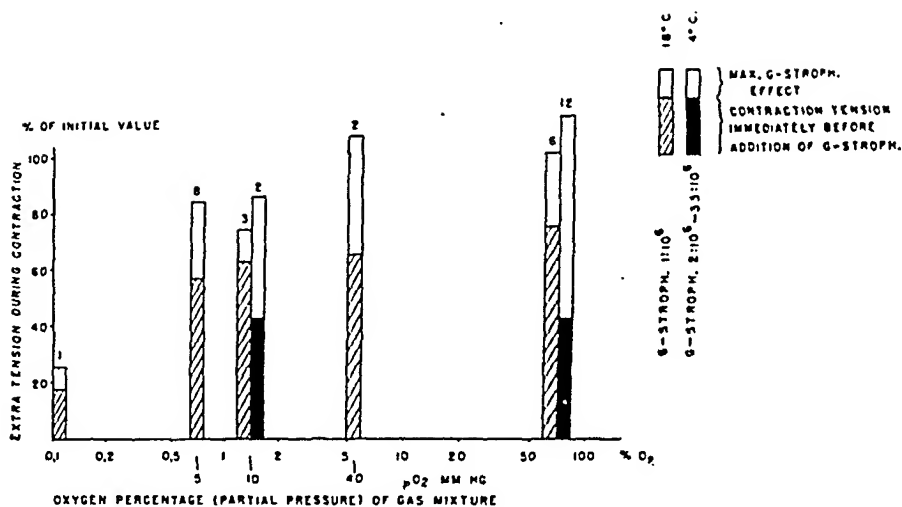


Fig. 1. Effect of g-strophanthin on extra tension during contraction. Means of several experiments as denoted by numbers.

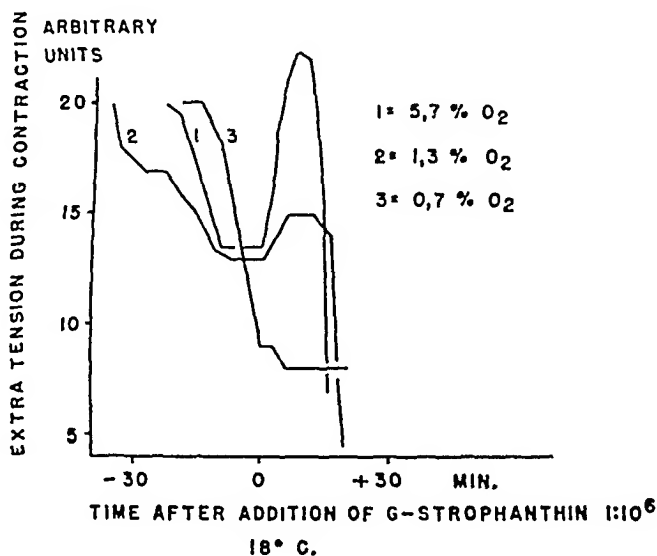


Fig. 2. Effect of g-strophanthin on extra tension during contraction.

et al. 1938). This was the case with our preparations, at least in most instances. In a small minority of our preparations this decline in contraction power was not observed even after a couple of hours. In a few preparations the contraction power fell to even a third of the initial value, although the oxygen supply was adequate.

The spontaneous fall in contraction power can be accelerated

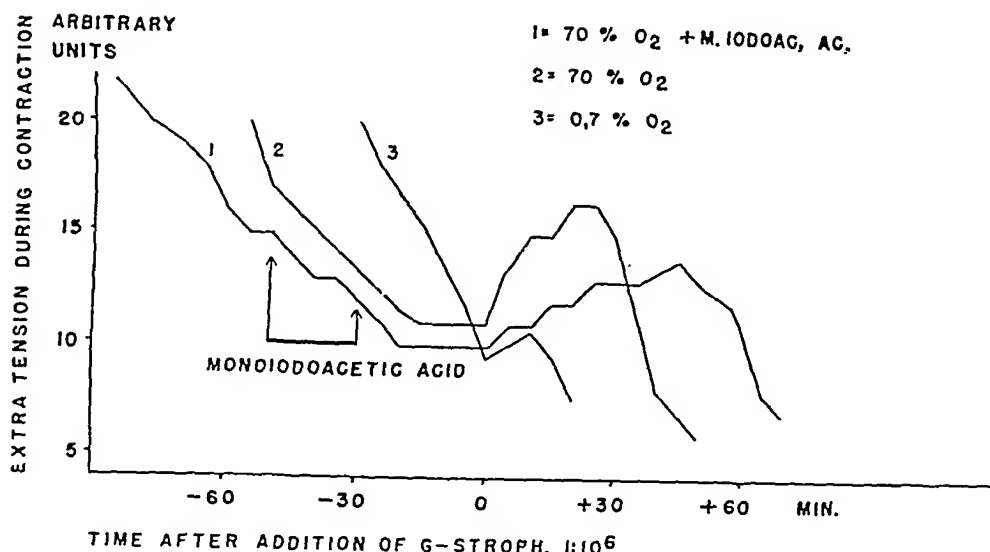


Fig. 3. Effect of g-strophanthin on extra tension during contraction of muscle treated with monoiodoacetic acid compared with contraction tension of untreated muscle 18° C.

by bubbling through the Ringer's solution the above mentioned gas mixtures with a low oxygen content. When the quantity of muscle tissue is small in comparison to the volume of the surrounding solution, as was the case in our experiments, the only factor governing the oxygen supply to the muscle will be the partial pressure of oxygen. The critical pO_2 , below which frog's ventricle cannot maintain normal activity, is 20–30 mm Hg, and the pO_2 , below which only anaerobic activity is maintained, is 5–10 mm Hg (CLARK et al., 1938).

G-strophanthin, added to the hypodynamic heart muscle preparation in Ringer's solution with an oxygen pressure of 40 mm Hg or more, in all cases augments the extra tension developed during contraction. By this increase in extra tension the contraction power of the preparation is restored to the initial value at the beginning of the experiment, which is assumed to be the "normal" value. Under the influence of g-strophanthin, diastolic tension remained unaffected until the appearance of the toxic phase, which manifested itself by a fall in extra tension during contraction together with a rise in diastolic tension.

In experiments with an oxygen pressure in the Ringer's solution of 10 mm Hg or less the effect of g-strophanthin is reduced, even if in most cases the extra tension during contraction is slightly rised. This rise, however, never restores the contraction

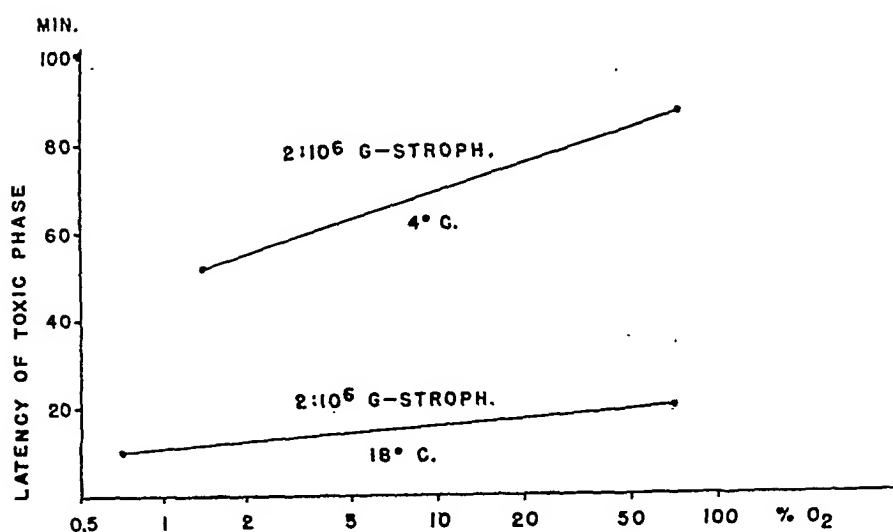


Fig. 4. Latency of toxic phase.

power to the "normal" value. At the lowest oxygen pressure, 1 mm Hg, there is hardly any perceptible effect of g-strophanthin. This difference between the action of g-strophanthin at high and low oxygen pressures is more marked at 18° C. than at 4° C. (fig. 1).

If the anoxic musculature after onset of the toxic effect of g-strophanthin is supplied with 70 % oxygen, extra tension during contraction usually rises very suddenly, even up to the "normal" initial value, but only if the toxic phase has just started. After some minutes the augmenting effect of oxygen usually passes off, but in some experiments it lasted for more than ten minutes.

Preparations treated with monoiodoacetic acid, where the glycolysis is nearly totally absent, continue responding to stimulation if supplied with a large amount of oxygen. In such preparations g-strophanthin produces only a moderate increase in the extra tension during contraction.

The effect of g-strophanthin 1 : 10⁶ at different oxygen pressures is shown in fig. 1. In fig. 2 and 3 extra tension during contraction is represented in relation to time. From these figures it is clear that there is quite a wide variance in experiments with 0.7 % O₂ at this concentration of g-strophanthin.

The time of onset of the toxic phase of g-strophanthin in a concentration of 2 : 10⁶ with different oxygen pressures and at

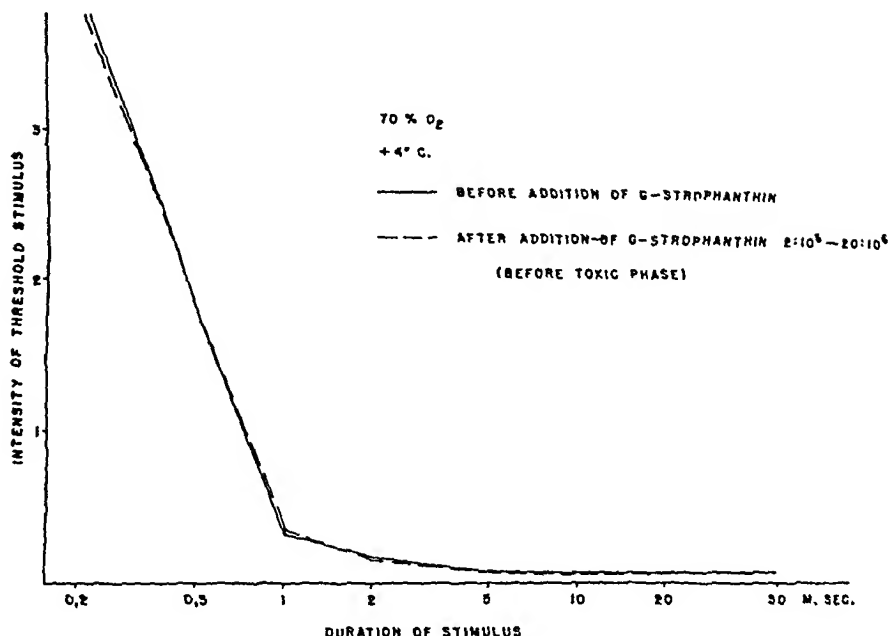


Fig. 5. Excitability of heart muscle preparation before and after addition of g-strophanthin at various concentrations. Mean of 6 experiments.

different temperatures is shown in fig. 4. The toxic phase has a tendency to appear earlier at low than at high oxygen pressures. This is not in accordance with the findings of WEIZSÄCKER (1913) who stated that in an isolated frog's heart treated with KCN the toxic effect of g-strophanthin appears later than in a normal heart.

The difference between the latency of action of g-strophanthin at 18° C and at 4° C as shown by fig. 4 suggests a temperature coefficient Q_{10} of about 3. This result is in accordance with earlier statements (WEESE 1936). In experiments with higher concentrations of g-strophanthin, however, we observed higher figures for Q_{10} , up to about 6.

We estimated the excitability under the influence of g-strophanthin. From fig. 5 it will be clear that no significant change was observed. This is in agreement with earlier investigations (WEESE 1936).

Summary.

Our observations indicate that:

1. G-strophanthin has a positive inotropic effect on hypodynamic heart muscle of the frog.

2. The rise in contraction power produced by g-strophanthin restores the extra tension developed during contraction to the assumed "normal" value and not higher.

3. If the partial pressure of oxygen surrounding the muscle is below a certain value, or if glycolysis is interfered with, g-strophanthin does not restore the extra tension during contraction to the "normal" value.

4. G-strophanthin does not influence the diastolic tension or the excitability before the appearance of the toxic phase.

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Polarity of Dark-Adapted Retinal on/off- Elements as a Function of Wave-Length.

By

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GERNANDT and GRANIT (1947) noted that the polarity of the retinal element, isolated by the micro-electrode technique depended upon its type. In order to determine the polarity a constant current was led through the retina of a dark-adapted decerebrated cat between silver-silverchloride electrodes in the nasal and temporal corners of its eye. The micro-electrode was located in the nasal half of the retina. The cathodal element responded to the make of the rheobasic current with nasal electrode cathode, the anodal element to the current with the same electrode anode. All on-elements proved to be cathodal, all off-elements anodal, the on/off-elements cathodal or anodal depending upon their off/on-ratio. The type of the element, whether an on-, off- or on/off-element, was nearly always determined with green light of wave-length $0.520\ \mu$.

In the present work merely on/off-elements have been included in the material. All the pure on-elements proved to be cathodal, all the pure off-elements anodal, as in the previous work. It was noted by GERNANDT and GRANIT (1947) that the more off-sensitive on/off-elements (dark adaptation, green test light) tended to be anodal, those of greater on-sensitivity cathodal. This suggested that polarity was a function of the off/on-ratio, introduced into the analysis by GRANIT and TANSLEY (1948) who established the enormous range of variation of this property of the complex on/off-elements. Further experiments (GERNANDT, 1948) proved

the off/on-ratio itself to be a function of wave-length. Thus arose the problem of how polarity and off/on-ratio are distributed for different colours. The present work deals with it.

Technique.

Decerebrated, fully dark adapted cats were used which had received 3—6 ml of a 20 % solution of urethane in order to eliminate eye and head movements. The polarization electrodes were placed alongside the bulb in the nasal and temporal cavities and fixed. The cornea and lens were removed and the micro-electrode inserted. The animal was left to dark adapt for not less than 1 hour with the micro-electrode in the eye. As to details of technique, see GERNANDT and GRANIT (1947).

Polarization took place over a resistance of 50,000 Ω . The elements responded to the make of currents from 0.005 mA upwards.

From our large Wright colorimeter (1946) the wave-length 0.510 (green), 0.650 (red) and 0.460 (blue) were selected for stimulation in order to determine the off/on-ratio at the threshold of illumination. A number of the 'green' off/on-ratios were determined with wave-length 0.520 μ .

Results.

The material has been collected in the course of various experiments with the micro-electrode technique all of which have been standardized to identical conditions. It has become a matter of routine always to determine the polarity of an element. Similarly measurements of the light threshold are often carried out, sometimes with green alone, sometimes with other wave-lengths, in order to determine the off/on-ratio. The present material consists of 321 well isolated on/off-elements. For 72 of them the thresholds were determined with both green, red and blue. In addition there were about 10 elements of indifferent polarity. One might have expected this to happen only for off/on-ratios around 1.0, but there was no definite correlation with the off/on-ratio.

Fig. 1 reproduces the distribution curves for the off/on-ratios of 196 elements tested with green, 105 with red and 100 tested with blue light. The white columns refer to anodal elements, the dark columns to cathodal ones. The ordinates are number of elements within a given logarithmic variation of the off/on-ratio on the abscissae. Low off/on-ratios are to the left, high off/on-ratios to the right of the point of equal sensitivity for 'on' and 'off'.

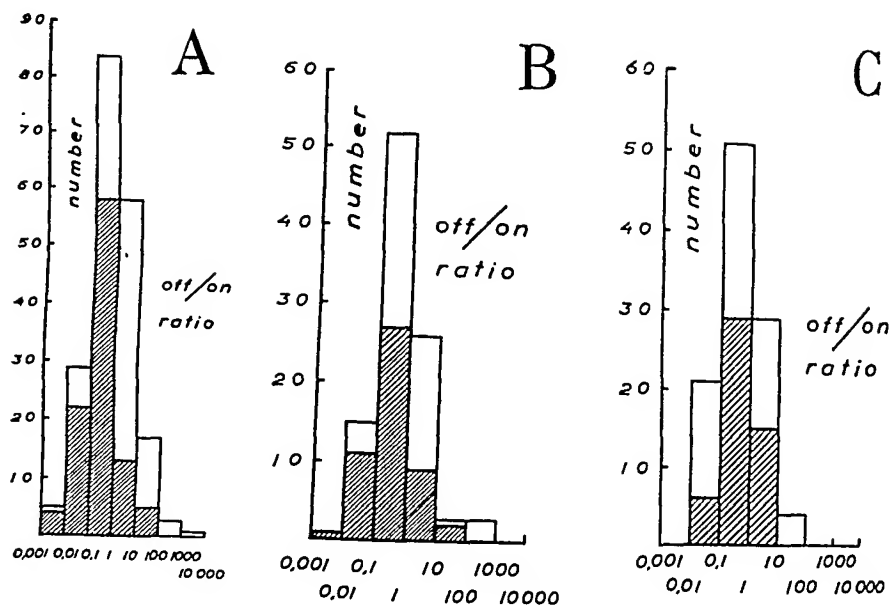


Fig. 1. The ordinates show the number of elements present within certain ranges of off/on-ratios which are grouped in logarithmic units of the abscissae A. Green, B. Blue, C. Red (see text).

A. *Green*: The diagram confirms for a larger material the results of GERNANDT and GRANIT (1947). There is a tendency of the anodal elements to group themselves among those of higher off/on-ratio whereas the cathodal elements behave in an opposite fashion.

B. *Blue*: There is a similar tendency of the anodal and cathodal elements to group themselves according to off/on-ratio though not so well marked as with green.

C. *Red*: There is no definite tendency for the anodal and cathodal elements to be distributed according to off/on-ratio.

Comment.

Is there any result with which to correlate these observations? For the 72 elements, measured with all three wave-lengths figures were available for a comparison of colour and off/on-ratios. The probability that the maximum of the off/on-ratio was in the red was 0.47, for the blue the corresponding probability was 0.39 and for the green only 0.18. This may be stated somewhat differently: in a sufficiently large material of on/off-elements the off-component tends to be relatively more sensitive to stimulation with red than with green. Similarly the on-component tends to be partic-

ularly green-sensitive and less red-sensitive. Blue occupies an intermediate position. The off/on-ratios for blue and red had previously been compared by GRANIT and TANSLEY (1947) who also had found red to be a more efficient stimulus for the off-component than blue.

Considering that the pure on-element is cathodal (GERNANDT and GRANIT, 1947) it is accordingly suggested that in dark-adapted cats the correlation between cathodality and low off/on-ratios (= high on-sensitivity) depends upon the same factors that make the pure on-elements cathodal. Since by several criteria the pure on-elements are rods (GRANIT, 1947) it is possibly a greater contribution of rod-activity that tends to make certain on/off-elements of low off/on-ratio cathodal. The red stimulus at wavelength 0.650μ falls practically outside the spectral region within which visual purple plays a rôle.

Summary.

The micro-electrode technique and the eye of the fully dark-adapted cat have been used for measurements of off/on-ratios and polarities of retinal elements tested with red, green and blue light.

With red stimuli anodal and cathodal elements are distributed symmetrically over the off/on-ratios, but, if the latter be determined with green or blue light, elements of lower off/on-ratio tend to be cathodal those of higher off/on-ratio anodal.

The results are correlated with the relative values for the off/on-ratios in the three wave-lengths.

This work has been supported by a grant from the Rockefeller Foundation to the Nobel Institute for neurophysiology.

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Phosphatase in Cats with Obstructive Jaundice.

By

JØRGEN B. DALGAARD.

Received 4 February 1948.

I. Serum Phosphatase.

Patients with obstructive jaundice as well as dogs and rabbits with experimentally produced bile stasis display increased serum phosphatase. In a previous paper (1947) the author has proved that the same is true for rats. It therefore seemed surprising that, according to CANTAROW, STEWART and MCCOOL, 1936, THANNHAUSER *et alii*, 1937, and FLOOD, GUTMAN and GUTMAN, 1937, among all investigated animals, only cats should not show such increase.

That problem is here submitted to a thorough new investigation which shows that the cat in fact also displays increased serum phosphatase following ligation of the common bile duct.

Earlier Experiments on Cats.

CANTAROW, STEWART and MCCOOL (1936) investigated 8 normal cats and 30 with ligated common bile duct. But in most animals only a single analysis exists and preoperative analyses were not made. Furthermore, 4 postoperative values really were increased, one of them considerably. The evidence of this material thus seems rather inconclusive.

THANNHAUSER, REICHEL, GRATTAN and MADDOCK (1937) stated that "in cats the serum phosphatase does not increase as it does in dogs". But as only two experiments were given, which

in fact showed a slight increase and further statements were missing, the results evade judgement.

The work of FLOOD, GUTMAN and GUTMAN (1937) based upon pre- and postoperative analyses in 10 cats with ligated common bile duct, is the most important to our problem. The authors say that the rise in serum phosphatase "is negligible in comparison with the marked elevations regularly observed following obstruction of the common bile duct in the dog and in man". In eight of the cats, however, the following increases from pre-operative to postoperative values were observed: 5—9, 1.5—4, 0.6—3.6, 0.8—5.7, 1—15.7, 2—5.6, 2.8—12.9 and 1.9—11.2. The present writer cannot agree that such increases are negligible.

Experimental. ¶

The investigation concerns 39 cats in 10 of which the common bile duct has been ligated. The phosphatase is estimated by a modified BUCH and BUCH method (1939) which in itself is a modified KING and ARMSTRONG method (1934) with phenylphosphate as a substrate buffered to pH 10. The hydrolysis time is extended from 15 to 30 minutes and all quantities of reagents are diminished to 2/5. The serum quantity, however, is only diminished to 200 mm³, which relatively means a doubling. That these alterations are allowed is seen from the curves in Fig. 1, which show that under these conditions there is complete proportionality between the amount of phosphatase (extinction) and amount of serum and time of hydrolysis respectively.

The readings are carried out on a Pulfrich Photometer with 1 cm cuvettes towards a blank, which is not hydrolysed, but immediately precipitated with trichloroacetic acid. Filter S 72 is used.

One unit of phosphatase is the amount of enzyme liberating 1 mg of phenol in 15 minutes. The phosphatase activity is expressed in units per 50 cc of serum and read on an extinction curve, made from known phenol solutions which are treated exactly as the serum samples. To express the phosphatase in ordinary BUCH and BUCH units (approximately = KING and ARMSTRONG units) all values have been divided by 4. The standard error of the mean of double estimations, which are always made, is 0.8 units.

The blood is obtained by heart puncture after light anaesthesia with coal-gas. It must be secured that the needle really is in the heart (pulsation) as an eventual pleural exudation from earlier heart punctures might be a source of error. In some cases blood is obtained from the tail or from the neck after decapitation. It is ascertained that samples from these different sources are identical in phosphatase

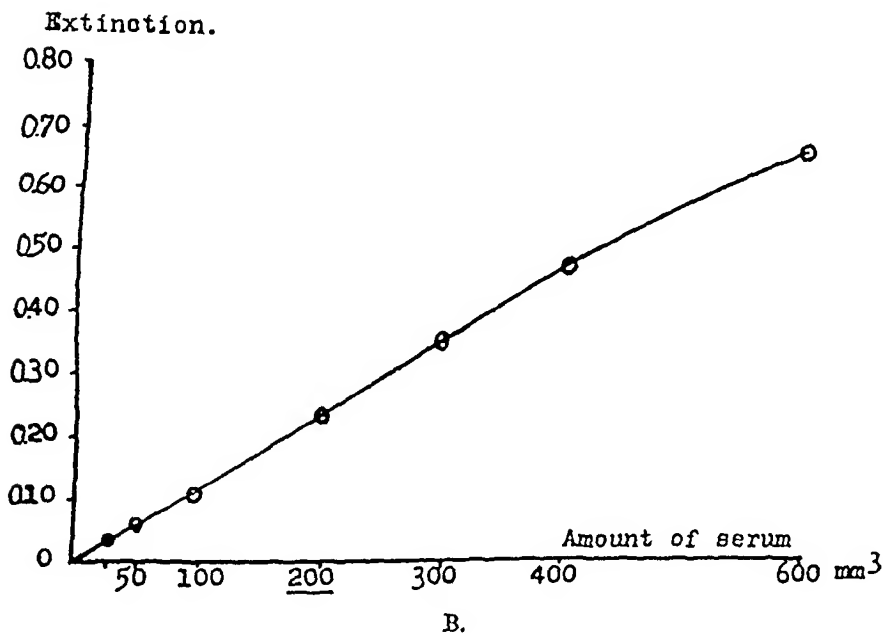
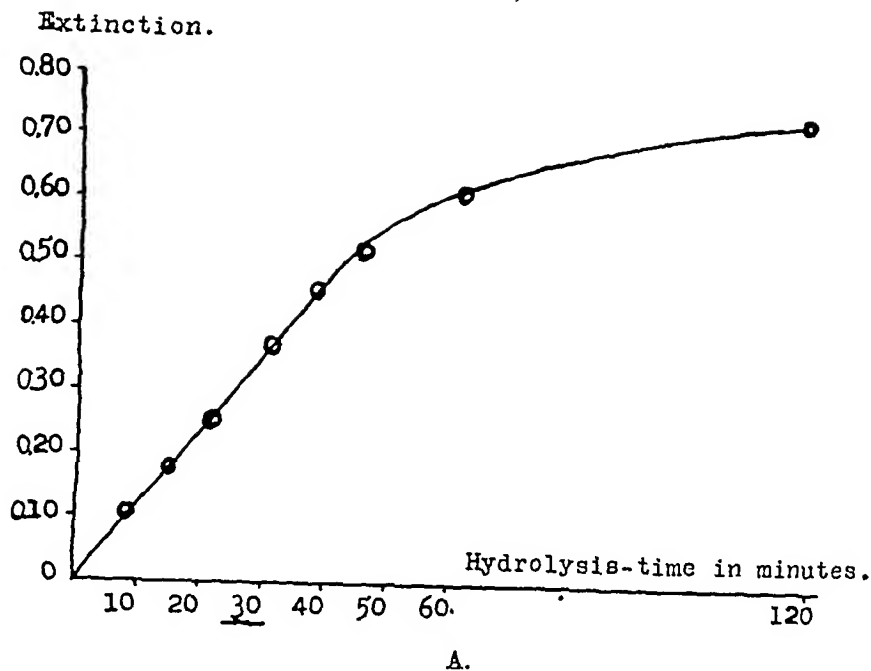


Fig. 1. The effect of alterations in hydrolysis-time (A) and amount of serum (B). The curves show that direct proportionality exists between phosphatase (extinction) and hydrolysis-time resp. amount of serum during the experimental conditions chosen. This again means that these are optimal.

amount. It is also controlled that the gassing had no influence on the serum phosphatase.

Hemolysis which in several experiments was proved to reduce the serum phosphatase (red blood corpuscles contain very little alkaline phosphatase), must be avoided through careful blood sampling and immediate centrifugation and separation.

Age variations: In accordance with the findings in man, dog, rabbit, rat and chicken (KAY 1930, BODANSKY, JAFFE and CHANDLER 1932, BODANSKY 1934, VERMEHREN 1938, DALGAARD 1947, COMMON 1936 and WIESE *and alii* 1939) the phosphatase level in young cats is higher than in grown-up animals. See table 1.

Table 1.

Age variations. The phosphatase level decreases with age. (The first 5 estimations originate from 3 kittens of the same litter. The 11 months value is a mean of 10 estimations from 4 cats of another litter).

Age	2 days	10 days	17 days	30 days	2 months	5 months	11 months	more than one year
Phosphatase	34	16	15	11	10	8	5.4	mean: 4

Sex variations: The mean of 53 estimations from 17 normal female cats is 4.3 ± 1.7 and the mean of 19 estimations from 7 males is 3.6 ± 1.4 . This suggests a slightly higher level in female cats. *Pregnancy* increases the serum phosphatase (Comp. VERMEHREN 1939). A cat showed 18 days before delivery a value of 6.4, and the last day 14.2. The normal level 42 days later, was 2.4.

Starvation causes a decrease in phosphatase in rabbits, guinea-pigs, dogs and rats (references by WACHSTEIN 1945), but according to VERMEHREN (1938) and BUCH (1942) not in man. The influence of starvation is here investigated in 11 experiments in 9 cats which first had a free supply of fish and milk and afterwards were starved for 40 hours. Most of them displayed a slight decrease during the starvation period, 2 cats, however, a slight increase. The mean before starvation was 4.6, after both 24 and 40 hours 4.2. Starvation is thus without practical importance in cats as opposed to the writer's previous findings in rats.

Normal values: The mean of 72 estimations in 24 normal, grown-up cats is 4.1 ± 1.7 phosphatase units. The variability is thus rather pronounced even in the same cat and the normal level is rather low, as compared to man, dog, rabbit, sheep and especially rat.

The operations are performed in coal-gas-ether anaesthesia. The common bile duct is doubly ligated with silk 1—1½ cm from the duodenum. (Concerning cats Nos. 11 and 26, see below.) Jaundice appeared in 2—3 days. The serum icterus index, which is normally about 5, reached a maximum in 11—15 days, after which a slight decrease began (see the stippled line in Fig. 3, which is a mean curve). The autopsy proved that the bile stasis was complete in all operated animals (except K₁₁, see below). The bile ducts were dilated, often to an enormous extent, as shown in Fig. 2.



Fig. 2. Photograph from the autopsy of a cat (K₁₂), which shows enormous dilation of the gallbladder (g. b.) and the bile ducts (b. d.), especially the common bile duct (c. b. d.), which was ligated 30 days previously.

Serum phosphatase following common bile duct ligation.

34 phosphatase estimations *before* and 71 *after* common bile duct ligation in 10 cats are made. One cat, K₂₇, died from the heart puncture the day after the operation and is omitted in the table. Excluding this animal *all ligated cats show an increased serum phosphatase after the operation*. In table 2 all the phosphatase estimations are given in a way that makes it possible to follow the phosphatase curve of each single cat. It is noticed that the postoperative values are somewhat higher than the preoperative ones. But while some animals, *e. g.* K₂₆ and K₃₃, show a rather regular curve, others have a less regular one. In K₂₁ only a slight increase is observed. Some very few values, as K₂₆ on the 12th day seem unlikely and are supposed to be erroneous, but are included for the sake of completeness.

Table 2.

Table of the pre- and postoperative phosphatase values in 9 cats with ligated common bile duct. In spite of the large variations an increase in phosphatase is obvious after the ligation.

Cat No.	Days before ligation				Days after ligation of the common bile duct																
	4	3-2	1	0	1	2	3	4	5	6	7	8-9	10-11	12-15	16-19	20-23	24-27	28-31	38-42		
9	2.9	2.6		4.7	3.3	2.0	7.5	18.7	21	14	14.9	23		36							
11	5.6	5.6	8.6		7.0	10.8	5.0				15.1		9.4	10.8	5.8	10.1	12 ?				
19		3.6		1.7	2.1	1.3		15				7.7	5.7	18.2	5.2	9.2	4.6	10.5 10.3			
21	3.6			7.0	4.5	4.5				8.4		5.4									
26	3.9	2.7	1.8				8.5			16	16		19	3.6 11	16.8	9.8 12.2		3.2	6.6 3.4 4.5		
28	5.1	4.0	2.8	3.0	1.0			7.2	7.9												
30	3.1	1.4	5.5	4.8			6.4			11		11.4									
31	2.4	4.3		6.1			1.4			11	5.9		8.5	8.2	13.6	8 7.5	5.5				
33	0.8	5.1	6.2	0.5	3.8			10			12		16.5	15	21						
Mean value of 72 analyses 4.1 ± 1.7					3.6	4.7	5.8	13.3		12.4		11.9	14.7	12.5	9.5	7.4	8.0	4.8			

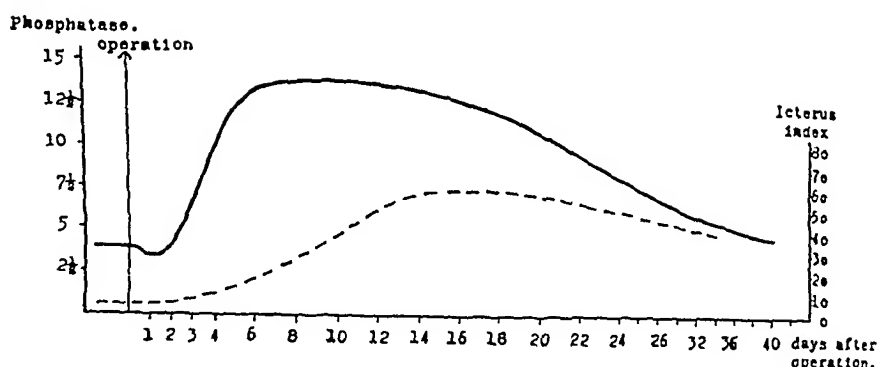


Fig. 3. Serum phosphatase after ligation of the common bile duct. The curve is a mean from 9 cats. The dotted curve is the mean curve of icterus index.

The last line in table 2 shows the mean values on the individual days or of a few days together. The best survey of the whole material, however, is given in the curve Fig. 3. The slight drop in phosphatase the day after the operation is probably an effect of starvation as the cats were starved only 18 hours previous to the operations. From the third day, however, an increase is noticed, which reaches a maximum about three times the normal level on the 6th day and remains at this level during the next two weeks. After the 20th day a slight decrease is noticed, which continues as long as analyses are made and about the 40th day has reached the normal level again. This observation, however, originates from a single animal only.

Some similarity exists between the phosphatase curve and serum icterus curve in Fig. 3 but no direct proportionality is at hand.

In two cats, K_{11} and K_{26} the common bile duct was ligated with a single thin catgut instead of silk. It was hoped that in some days this catgut would rupture, thus allowing the bile to flow again. In K_{26} this did not happen, the course and the autopsy showed that the bile stasis was complete and lasting. Otherwise in K_{11} . At first this animal reacted quite in the usual way. The appetite had gone, the animal lost weight, became jaundiced, dirty and dull. 14 days after the operation the icterus index was 60. The phosphatase had increased as in the other ligated animals to a maximum of 15 units.

On the 17th day, however, the icterus index had suddenly dropped till $17\frac{1}{2}$ and the animal was obviously much better. Soon after, the visible jaundice decreased, the cat began to eat more, gained weight and became cleanly. Normal icterus index

was reached already on the 20th day — in striking contrast to the other animals operated on. The cat was killed on the 45th day. At the autopsy the gall bladder seemed normal. The common bile duct was neither dilated nor strictured but could be probed till the duodenum, whose contents were bile coloured. No doubt exists, therefore, that between the 14th and the 17th day the catgut ligature had ruptured and a normal bile-flow been re-established.

A few days later a gradual decrease in phosphatase occurred, which reached the normal level on the 29th day. As the drop in icterus index was rather abrupt this is another evidence against a direct proportionality between the two.

Conclusion on serum phosphatase.

In opposition to the conclusions of previous authors on cats, the writer reaches the following conclusions from his material, which has been investigated much more thoroughly than earlier ones: *The cat shows increased serum phosphatase following ligation of the common bile duct.* This pathophysiologic reaction is of the same type as, although less pronounced than, that of all other investigated animals. The quantitative difference may be associated with the normal low phosphatase level of the cat, that again may be caused by the dietary habits of this animal, which is a milk and protein-eater as opposed to the other animals and man, being chiefly carbohydrate-eaters.

II. Phosphatase in Bile.

Bile has been secured for phosphatase analyses at the autopsy, immediately after death, of 3 normal cats and 7 cats with ligated common bile duct. The latter include two nephrectomized cats, K₅ and K₃₄. (See section IV.) The bile was diluted with a 0.9 per cent saline solution and analysed in the way described above, but only 50 mm and 15 minutes hydrolysis time was used. The figures are converted into the usual units and are given in table 3.

Bile normally contains very large amounts of phosphatase. Following ligation of the common bile duct the phosphatase activity falls considerably. The minimum in these cases was found 7—9 days after the operation. Later on, while the serum phosphatase approaches the normal level, the biliary phosphatase seems to rise again.

Table 3.

Phosphatase in bile, which normally is very high, decreases following ligation of the common bile duct.

Cat No.	27	5	34	30	21	33	19	10	14	16
Days after operation.....	1	2	3	7	9	9	30	Normals		
Phosphatase in bile.	144	154	26	9.6	12	62	80	200	95	208

FLOOD, GUTMAN and GUTMAN also report decrease in biliary phosphatase following ligation of the common bile duct but in their cases no re-increase was observed.

III. Phosphatase in Urine.

Urine from dog and man contains only inconspicuous amounts of alkaline phosphatase both normally and following bile obstruction. In most normal cats, however, FLOOD, GUTMAN and GUTMAN found phosphatase and after ligation of the common bile duct an increase above the normal level was observed in 5 of 12 investigated cats. These observations made the authors assume that the kidney of the cat has the ability to clear alkaline phosphatase. This question will be discussed again in the next section.

Experimental.

Urine has mostly been obtained by catheterization in female cats whose urethra ends about $\frac{1}{2}$ cm up in the anterior vaginal wall. The phosphatase was estimated as in bile (but without dilution) and converted into the usual units. (All methods depending upon phosphorus analyses are less convenient for urine because of its appreciable and varying phosphorus content).

The amount of phosphatase in the cat's urine proved rather high and variable. 24 estimations from 14 different *normal cats* gave a mean of 48 ± 30 units. Following ligation of the common bile duct 31 estimations from the 1st to the 45th day after the operation of 9 different cats are available. The variation is appreciable, but no characteristic fluctuations are observed. The mean of all postoperative values is 37 ± 27 units. *The present*

material thus does not reveal any sign of increase of urinary phosphatase following bile obstruction.

Investigations on sediment and supernatant urine were sometimes made after ordinary centrifugation at a rate of 3,000 revolutions per minute for 10 minutes. The sediment constantly had a higher phosphatase activity than the total urine, which again had a slightly higher activity than the supernatant urine. But the total amount of phosphatase was always largest in the supernatant urine as the sediment was scanty. (Comp. BREEDIS, FLOREY and FURTH, 1943.) Histochemical investigation according to GOMORI has been performed on the sediment, which mostly consisted of desquamated epithelial cells of the bladder containing phosphatase. A few leukocytes and tubular cells were also met with.

IV. Serum Phosphatase after Bile Obstruction and Nephrectomy.

Until now it was believed, as previously mentioned, that the cat does not increase the serum phosphatase after ligation of the common bile duct. Furthermore, the high urinary phosphatase and the alleged increase hereof during bile obstruction was known.

GOMORI, 1939, with his histochemical method demonstrated the presence of phosphatase in the glomeruli of cats but not in other species. This observation has been confirmed by WILMER (1944) and the author can also sustain it.

A combination of these peculiarities might lead to the following hypothesis which was hinted at by MOOG (1946): The cat has the ability to clear alkaline phosphatase and, after bile obstruction, to compensate an expected increase in serum phosphatase through increased output via the kidneys.

Although the chief arguments for this hypothesis have been refuted already, it might yet be of some relative validity. This section intends to give a clear experimental answer to that.

If the kidneys are excluded on cats with ligated common bile duct, a distinct rise in serum phosphatase must be expected if the hypothesis is right. If, on the other hand, such rise does not occur, the hypothesis must be abandoned.

Previous experiments in this field is only performed by FLOOD, GUTMAN and GUTMAN who ligated the ureters of two cats which previously had their common bile ducts ligated. Increase in phosphatase was *not* observed. But only one, resp. two analyses were obtained, and not later than the second day. So the authors dare not draw any conclusions from these two experiments.

Experimental.

Nephrectomy is more appropriate than ligation of the ureters which does not completely exclude the kidneys. In this investigation 10 cats have been used. Concerning blood sampling, phosphatase estimation and anaesthesia, see section 1. In 7 cats the common bile duct was doubly ligated with silk and the kidneys decapsulated and removed; in three controls (K_{36} , K_{37} and K_{39}) the kidneys were removed but the common bile duct not ligated. The cats were starved for 18 hours prior to the operation. Afterwards they got milk but hardly drank any. On the second day they became seedy, on the third day more seedy with vomitings. Blood urea exceeded 300 mg per cent on the third day and 400 mg per cent on the fourth day after operation. When the cats were so ill that they could not stand, they were killed (on the third or fourth day). At the autopsy complete bile stasis was found in all ligated animals. In accordance with this the serum icterus index had increased from the normal (about 5—6) till about 10—15. One animal had developed an abscess in one of the kidney beds and was left out.

Results: From table 4 it appears that all pre- as well as post-operative values in the first four, most carefully investigated animals, are within normal limits. The single increased value in K_5 two days after the operation, cannot be trusted too much, as no less than 3 different analyses next day are normal.

The investigation thus shows that the serum phosphatase does not increase after common bile duct ligation and simultaneous nephrectomy. The hypothesis which was tried, must thus be abandoned as incorrect: The cat does not compensate a rise in serum phosphatase by means of phosphatase excretion via the kidneys. It shall be added that these results concern the active enzyme only, nothing is shown concerning possible pro-enzymes.

In connection with these experiments it is of interest to study what influence uremia may have on serum phosphatase. The controls in table 4 indicate that a slight decrease occurs. According to PYLE, FISHER and CLARK (1937) creatine or creatinine do not inhibit phosphatase. Urea, therefore, must be tried.

In vitro experiments with urea: In serum samples from 3 different cats the phosphatase activity was estimated after addition of pure urea in different concentrations. An inhibition began at 100 mg per cent of urea and reached 30 per cent at a concentration of 300 mg per cent of urea and 40 per cent at 800 mg per cent.

Table 4.

Serum phosphatase before and after nephrectomy with (the first 6) or without (last 3) simultaneous ligation of the common bile duct. No phosphatase increase, but on the contrary a slight drop is observed in most cases.

Cat No.	days before operation			days after		
	3	2	1	1	2	3
15		4.1	4.9	5.7	2.6	2.5
17	4.3	4.8	4.0	2.3	3.0	3.1
18	4.3	4.3	3.2	1.7	4.1	6.6
34	5.2	4.2	7.0	2.4	3.8	2.5
5		5			14 (?)	4
7			8	8	6	0
36	2	0.9	2.5	4.0	2.0	0.7
37		4.0	4.0	1.7	2.0	
39	1.0	4.0	—	1.5	1.0	1.0

This finding may explain why the double-operated cats in table 7 do not display increased phosphatase even on the third day, which should otherwise be expected according to the results in section I.

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Summary.

It has been maintained that the serum phosphatase of cats does not increase after common bile duct ligation as it does in other animals and man, but this assumption seems weakly supported.

The present material comprises 39 cats in 10 of which the common bile duct has been ligated. The phosphatase is estimated by a

modified BUCH and BUCH (KING and ARMSTRONG) method. The variability of serum phosphatase is appreciable but a few normal variations are noticed: Young cats have a high level. During pregnancy the phosphatase increases. The mean value for female cats is slightly higher than for males. 40 hours of fasting affords only an inconspicuous decrease.

All cats with ligated common bile duct display an increase in serum phosphatase which begins on the 3rd day, reaches a maximum between the 4th and 17th day and then gradually decreases again. (See the mean curve, Fig. 3.) In one cat the bile flow was re-established after two weeks of bile-stasis and the serum phosphatase dropped to the normal level within a few days.

Phosphatase in bile which is normally very high, decreases after common bile duct ligation.

Urinary phosphatase vary very much normally. In the present material it does not increase during bile-stasis. The hypothesis that the cat is able to compensate an increase in serum phosphatase by an increased output through the kidneys must therefore be abandoned. This is in keeping with the findings in cats with simultaneous common bile duct ligation and nephrectomy. Such animals do not show increase of serum phosphatase.

In vitro experiments show that urea inhibits phosphatase.

The investigation shows that with regard to phosphatase during obstructive jaundice, the cat differs less from other animals than was previously supposed.

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The Effect of Essential, Synthetic Amino Acids on the Growth of Rats.

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It has been shown by ROSE (1938) that for the normal growth of rats the ten amino acids, arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophane and valine are essential. In these tests a mixture containing all the amino acids occurring in casein, except hydroxyglutamic acid and serine, was used. From this mixture one of the amino acids was removed and, if there was no change in the growth, the amino acid in question was regarded as dispensable; but, if the result was a decrease or a poorer increase in weight, the corresponding amino acid was considered not dispensable or essential. After this grouping of the amino acids, it was naturally of interest to see how far the growth of the rats could be promoted solely with the 10 essential amino acids. According to ROSE (l. c.), the rats grow equally well on a mixture with the 10 essential amino acids as on a mixture comprising all the 19 amino acids. Precise data regarding the results of these experiments have not been given by this author. In his tests, however, ROSE (according to a personal communication of 1947) never used synthetic amino acids solely.

ALBANESE and IRBY (1943) made experiments on growing rats with an amino acid mixture containing l-tryptophane, l-lysine, l-cystine, d-histidine, d-arginine, l-leucine, an l-leucine-isoleucine mixture, dl-valine, dl-methionine, dl-phenylalanine and dl-threonine. The result was a decrease of the body-weight of growing rats, despite of a positive nitrogen balance. The concentration of

the amino acid mixture in the diet was 14.7 %. If the concentration was increased to 29.4 % the rats markedly decreased in weight and half of them died within five days. From this the authors inferred that the racemic forms of the amino acids are toxic.

KINSEY and GRANT (1944) criticize the above-mentioned investigations of ALBANESE and IRBY (l. c.), pointing out manifest sources of error in these tests. They made corresponding investigations with the 10 essential amino acids solely. The amino acid mixture contained the following forms: l-arginine, l-histidine, dl-isoleucine, l-leucine, l-lysine, dl-methionine, dl-phenylalanine, dl-threonine, dl-tryptophane, and dl-valine. With a concentration of 5.8 and 11.6 % of this amino acid mixture in the diet a satisfactory growth was obtained in rats.

Corresponding results are reported by MARTIN (1944). He tested an amino acid mixture consisting of l-arginine, l-histidine, dl-isoleucine, l-leucine, l-lysine, dl-methionine, dl-phenylalanine, dl-threonine, l-tryptophane, dl-valine. With a concentration of 16 % of this mixture in the diet, a growth of ca. 1 gram per day in rats weighing 50 grams was obtained.

ALBANESE and IRBY's (l. c.) results must thus be considered to be erroneous, and it may be regarded as definitely ascertained that growth in rats can be promoted with the 10 essential amino acids solely. In the above-reported tests the natural isomers and racemic forms were used alternately. It has been shown that certain isomers of the amino acids cannot be utilized by the body. Thus, the unnatural isomers of isoleucine, leucine, lysine, threonine and valine cannot be used for the syntheses of body-protein, whereas the two isomers of histidine, methionine, phenylalanine and tryptophane can be utilized by the body (ROSE 1938). The question then arises whether the isomers that cannot be utilized by the body are toxic or harmless for the organism. Investigations on this subject have not previously been made. Should it be so that the organism can develop on a diet containing a mixture of the 10 essential amino acids solely, supplied in the racemic forms, this would signify that growth can be obtained with purely synthetic amino acids. In the chemical synthesis of amino acids racemic compounds, as we know, are always obtained.

In the investigation reported below, it is shown that with the 10 essential amino acids, synthetically produced, as the sole source of nitrogen, growth in rats can be promoted.

Experimental.

Amino acids: All the amino acids used in these experiments were synthesized by the author in accordance with the following methods:

dl-arginine HCl. The basis of the production was cyclohexanol, which was oxidized, with nitric acid, to adipic acid (ELLIS 1946). The adipic acid was decarboxylated to cyclopentanone (THORPE and KON 1946), which was converted into δ -benzoyl-ornithine via cyclopentanone oxime, piperidone, δ -benzoylamino-n-valeric acid, δ -benzoylamino- α -bromo-n-valeric acid (FOX, DUNN and STODDARD 1941). By benzoylation of δ -benzoylornithine, ornithuric acid was then obtained. From ornithuric acid, via α -benzoylornithine and α -benzoylarginine, arginine mono-hydrochloride according to the method of SÖRENSEN, HÖYRUP and ANDERSEN (1911/12) was produced. — From 230 g cyclohexanol 14 g dl-arginine HCl (2.9 % of the theoretical amount) were obtained.

dl-histidine HCl. In one series the synthesis was based on citric acid, which was converted into acetone dicarbonic acid (v. PECHMANN 1891). The latter was treated with sodium nitrite, thereby forming diisonitrosacetone (v. PECHMANN and WEHSARG 1886). By reduction, diaminoacetone was then obtained (KALISCHER 1895). From the latter product, dl-histidine mono HCl, according to the method of PYMAN (1911) was then produced, via 2-thiol-4(or 5)-aminomethylglyoxaline, 4(or 5)-hydroxymethylglyoxaline, 4(or 5)-chloromethylglyoxaline, ethyl-4(or 5)glyoxalinemethylchloromalonate, α -chloro- β -glyoxaline-4(or 5)-propionic acid. In this process, 12 g dl-histidine HCl (0.3 % of the theoretical amount) were produced from 4.5 kg of citric acid.

In subsequent synthesis 4(or 5)-hydroxymethylglyoxaline was produced from saccharose in accordance with the methods of ALBERTSON and ARCHER (1945) and TOTTER and DARBY (1944), whereupon the product was converted into 4(or 5)-chloromethylglyoxaline according to the method of PYMAN (1911). By condensing with ethylacetamidomalonate and following hydrolysis and decarboxylation dl-histidine di-HCl was then obtained (ALBERTSON and ARCHER 1945). From 171 g saccharose 3.5 g dl-histidine di-HCl were obtained (6.5 % of the theoretical amount, reckoned from hydroxymethylglyoxaline HCl).

dl-isoleucine. Produced from sec. butyl alcohol, which was converted into sec. butyl bromide (GOSHORN and BOYD 1946). By condensing sec. butyl bromide and diethyl malonate, bromination, hydrolysis, decarboxylation and amination, dl-isoleucine was then obtained (MARVEL 1941). From 135 g sec. butyl alcohol, 34 g dl-isoleucine were produced (14 % of the theoretical amount).

dl-leucine. From isobutyl alcohol, isobutylbromide (GOSHORN and BOYD 1946) was produced, whereupon a similar synthetic process as in the production of dl-isoleucine was applied. From 280 g iso-

butyl alcohol 70 g dl-leucine were obtained (14 % of the theoretical amount).

dl-lysine HCl. It was produced from cyclohexanone via cyclohexanone oxime, ϵ -benzoylaminocaproic acid, ϵ -benzoyl- α -bromocaproic acid and dl- ϵ -benzoyllysine (ECK and MARVEL 1946). The output was 66 g dl-lysine HCl from 558 g cyclohexanone (6 % of the theoretical amount).

dl-methionine. The syntheses was based on β -chloroethylmethyl sulphide produced from hydroxyethylmethyl sulphide (KIRNER and WINDUS 1946), obtained by condensation of methyl mercaptan and ethylene chlorohydrin (WINDUS and SCHILDNECK 1946). Methyl mercaptan was produced from methyl isothiouraea sulphate, obtained from thiourea and methyl sulphate (SCHILDNECK and WINDUS 1946). β -chloroethylmethyl sulphide was condensed with ethyl sodium phthalimidomalonate into ethyl 1-methylthiol-3-phthalimidopropane-3,3-dicarboxylate, which compound was hydrolyzed and decarboxylated. Methionine was so obtained via 1-methyl-thiol-3-phthalimidopropane-3,3-dicarboxylic acid (BARGER and WEICHSELBAUM 1946).

The ethyl phthalimidomalonate was produced from ethylbromomalonate and potassium phthalimide (OSTERBERG 1946). Ethylbromomalonate was synthesized in accordance with the method of PALMER and MCWHERTER (1946). Potassium phthalimide was produced by the method of SALZBERG and SUPNIEWSKI (1946) from phthalimide, which, in turn, was obtained from phthalic anhydride and NH_4OH (NOYES and PORTER 1946). From 230 g thiourea and 610 g phthalic anhydride, 100 g methionine were obtained (22 and 16 %, respectively, of the theoretical amount).

dl-phenylalanine. From benzylchloride via diethylbenzylmalonate, α -bromo- β -phenylpropionic acid, phenylalanine was synthesized (MARVEL 1941). From 211 g benzylchloride 100 g dl-phenyl-alanine were obtained (40 % of the theoretical amount).

dl-threonine was prepared according to CARTER and WEST (1937) from crotonic acid via α -bromo- β -methoxy-n-butyric acid. From 516 g crotonic acid 80 g dl-threonine were obtained (11 % of the theoretical amount).

dl-tryptophane. The basis of the synthesis was indole via gramine, the methiodide of the gramine (SNYDER, SMITH and STEWART 1944), ethyl- α -carbethoxy- α -acetamido- β -(3-indole)propionate, α -acetamino- α -carboxy- β -(3-indole)propionic acid (SNYDER and SMITH 1944). From 39 g indole, 26 g dl-tryptophane were obtained (38 % of the theoretical amount).

dl-valine. The production was based on isovaleric acid via α -bromo-isovaleric acid (MARVEL 1940). From 2,634 g isovaleric acid 880 g dl-valine were obtained (27 % of the theoretical amount).

Diet. As a nitrogen-free diet, a mixture of the following composition was used. The amount of nitrogen in the different constituents was determined according to the macro-Kjeldahl method (PETERS and VAN SLYKE 1932).

	Grams	Nitrogen percentage in the constituents
Dextrin	69	0.002
Wheat germ oil	1	0.004
Cod liver oil	2	0.02
Adeps suillus	19	0.001
Salt mixture (OSBORNE and MENDEL 1919)	4	0.006
Total	95	0.002 % N.

To this diet was then added a mixture of the synthetic essential amino acids with the following composition.

	Grams	Percentage ¹
dl-arginine HCl	2.6	8.3
dl-histidine HCl	1.2	3.6
dl-isoleucine	4.0	15.0
dl-leucine	4.0	15.0
dl-lysine HCl	2.8	8.4
dl-methionine	2.0	7.5
dl-phenylalanine	1.6	6.0
dl-threonine	3.2	12.0
dl-tryptophane	0.8	3.0
dl-valine	5.6	21.0
	27.8	

For neutralization of the hydrochlorides, 2.7 g NaHCO_3 per 27.8 amino acid mixture were added.

The experimental diets were thus obtained by mixing the amino acid mixture and the nitrogen-free diet. The following concentrations were used: 10, 20, 30 and 40 % (corresponding to 9.6, 19.2, 28.8 and 38.4 %, respectively, if the HCl in the hydrochlorides is subtracted).

Method. As experimental animals, white female rats with a weight of 44.5 to 50.4 grams were used. They were allowed to eat as much of the food as they pleased. Water was also given *ad libitum*. They daily received, in addition, 5.2 mg of a vitamin mixture composed as follows:

	mg
Aneurin	30
Riboflavin	50
Nicotinic acid amide	1,000
Pyridoxine	30
Ca-dl-panthotenate	100
Inositol	1,000
Choline chloride	3,000
Total	5,210.

¹ The percentages reckoned after subtraction of the amount of hydrochloric acid occurring as hydrochloride.

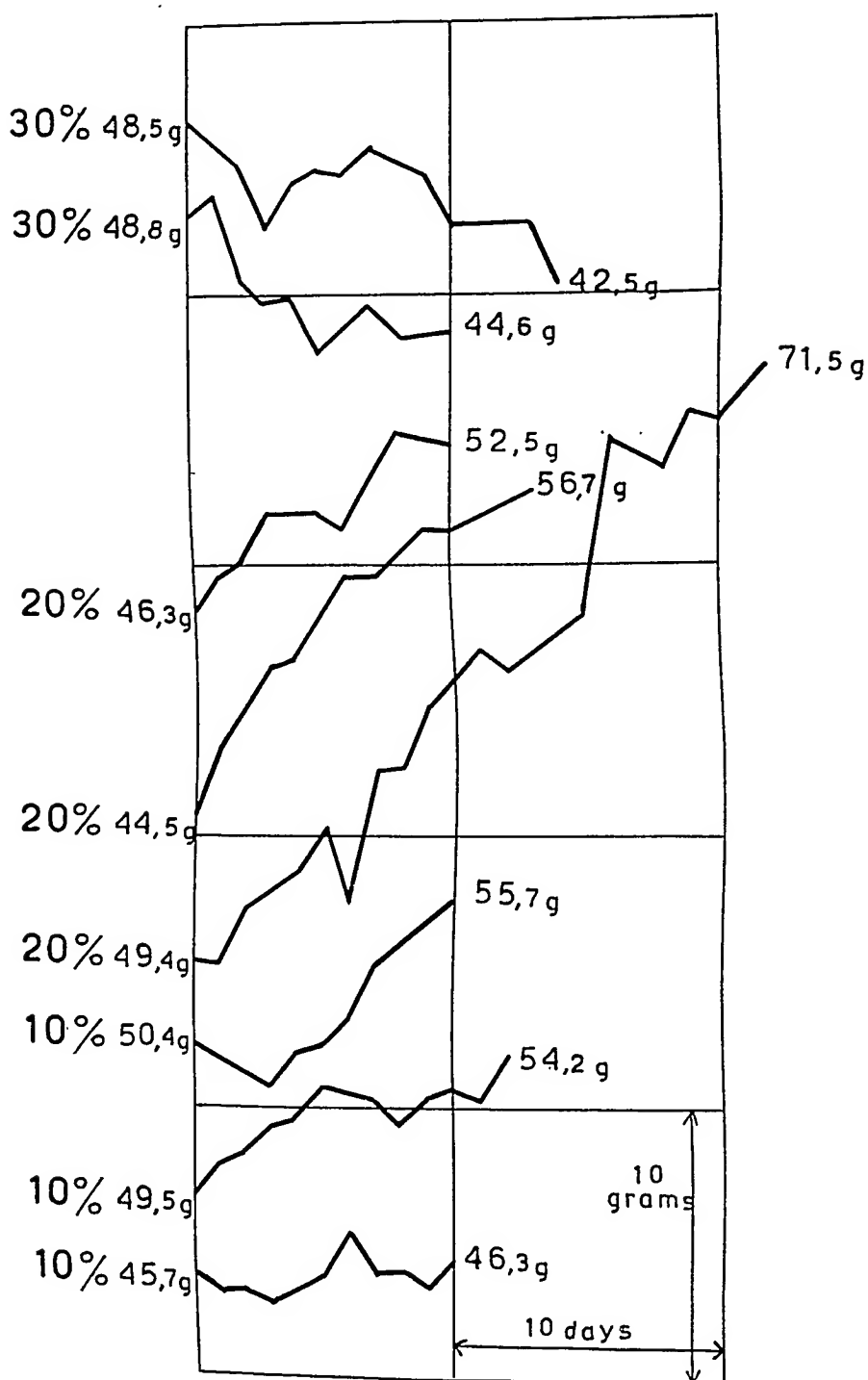


Fig. 1. The growth of rats with synthetic amino acids. The abscissa indicates the number of days, and the ordinate the body-weight. The concentration of the amino acid mixture in the diet is shown in front of each curve. At the beginning and end of each curve the initial and final weight, respectively, are indicated.

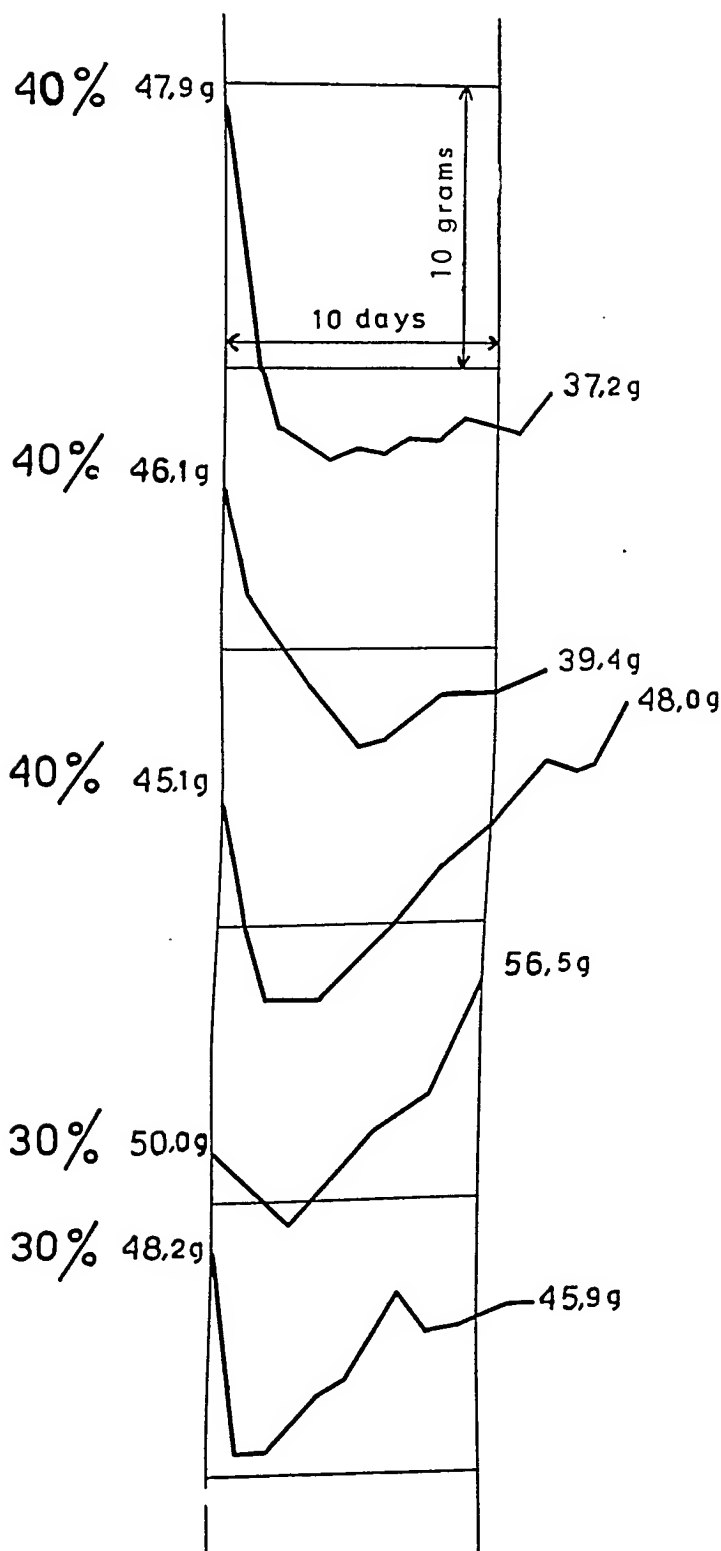


Fig. 2. The growth of rats with synthetic amino acids. Notations the same as in Fig. 1.

During the experiment the rats were kept in network metal cages placed in a room with a regulated temperature of 23—24° C. The experimental period varied between 10—22 days, the discontinuance of the tests being invariable due to shortage of amino acids. The animals were as a rule weighed daily at 9 a. m.

In fig. 1 and 2 the curves showing the weight of the rats on the different diets are traced.

Results: It is seen from fig. 1 and 2 that on a diet containing 10 % of a mixture of synthetic amino acids the rats increase in weight. The increase per the first 10 days averages 3.3 ± 1.4 grams. On the 20 % diet the increase in weight is better and surer, averaging 8.9 ± 1.5 grams per the first 10 days. But when the rats were put on a diet with 30 % amino acids, they showed in 3 cases of 4 a decrease in weight. The average decrease (in all 4 cases) was 1.1 ± 2.5 grams within the said time. If the rats were fed a diet containing 40 % of amino acids the result was a decrease in weight during the first 10 days averaging 6.3 ± 2.7 grams. One of the rats, however, seems to be able to increase its weight on the 40 % diet, as the weight curve after the first 10 days raises above the initial value.

These results thus indicate that solely with the 10 essential amino acids, synthetically produced, it is possible to maintain growth in rats if the amino acids are administered in certain concentrations in the diet (10 and 20 %). If the concentration is increased (up to 30 and 40 %), a decrease in weight results, instead.

Discussion.

The above-reported tests completely support the previous investigations, in which it has been shown that with the 10 essential amino acids solely, it is possible to promote growth in rats. They moreover show that even if the racemic compounds of these amino acids are used, growth will result. Thus it could be stated that the unnatural isomers of the essential amino acids are rather harmless as it was possible to maintain growth in rats if the amino acids were given in a concentration of 10 and 20 % of the diet. There seems, however, to be some toxicity of these racemic forms, as it was found that at higher concentrations (30 and 40 %) a decrease in weight occurred. Which of the amino acids or isomers produce this effect cannot be ascertained without a very thorough investigation. Naturally, the possibility that the synthetic amino

acids are chemically impure cannot be completely ruled out. This, however, seems to the author extremely improbable, as all the amino acids after they had been produced and been found to have the corresponding physical properties (melting point, crystalline form) were recrystallized.

Summary.

The author has studied the growth of rats which as the sole source of nitrogen had received a mixture of the racemic forms of the 10 essential amino acids, synthetically produced. As nitrogen-free diet, a mixture containing merely 0.002 % N was used.

On a diet containing 10 % of this amino acid mixture a growth averaging 0.33 ± 0.14 grams per day was obtained.

On a 20 % diet the growth of the rats averaged 0.89 ± 0.15 grams per day.

On the other hand if the rats received 30 and 40 % amino acids in the diet, a *decrease* in weight, averaging 0.11 ± 0.25 and 0.63 ± 0.27 grams per day respectively, resulted. Thus, it must be considered that these synthetic amino acids in their racemic forms, contain some toxic matter, but exactly whence it emanates has not yet been ascertained.

The synthesis of the 10 essential amino acids is briefly described.

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A Method for the Determination of Arginine in Urine and Serum with Remarks on the Excretion of Arginine in Humans.

By

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This method was worked out in the intention of trying to examine the reabsorption of arginine in the kidneys. A comparison of the renal reabsorption processes for several aminoacids has been made previously by PITTS (1944) and USSING (1945); their results will be discussed later. As, however, most of the experiments which will be described here were made just after the war, the lack of chemicals excluded a real examination of the problem of reabsorption, so the experiments regarding this are very few and only of an orientating kind and mainly given to show the application of the method. At the same time not much literature was available, so not until later it was discovered that in the meantime a similar method had been worked out by ALBANESE and FRANKSTON (1945). These authors determined the true arginine as the difference of the apparent arginine values of an aliquot treated with permutit and one not so treated, as they did not succeed in eluting the adsorbed arginine quantitatively from the permutit. The present method like that of ALBANESE and FRANKSTON (l. c.) is based upon the reaction of SAGAGUCHI: Arginine gives a red colour of unknown structure in alkaline medium when treated with α -naphthol and hypochlorite. The reaction is not quite specific for arginine as it is given by related compounds especially glycocyanine, methylguanidine and ammonia. Of these substances glycocyanine and ammonia probably play the most important part as methylguanidine according to most statements (GUGGEN-

HEIM, 1940) only occurs as a trace in urine under not pathological conditions. But this method differs from the above mentioned in the way that the separation of arginine from glycocyamine was made by adsorption of the arginine in 70 % alcoholic solution on an alkaline column of Al_2O_3 [prepared according to WIELAND (1942)] from which arginine could be eluated with 1/10 n HCl. In the production of the colour ALBANESE and FRANKSTON (l. c.) used a dilute sodium hypochlorite solution, which had proved to give the greatest stability and lowest blank. Here is used a potassium hypobromite solution prepared according to MACPHERSON (1942), and when the colour was produced as described later it gave reproducible values.

Experiments and Results.

The method for urine analyses is the following: About 5 g Al_2O_3 (Merck) is dispersed in 10 ml 70 % ethanol and a column about 80 mm in height is made in a tube with a diameter of 10 mm. The sample, 4 ml urine is made neutral to lithmus, and 96 % ethanol is added until the total volume is 15 ml. The alcoholic solution is gently sucked through the column, and after that 30 ml 70 % ethanol. When the column has been sucked practically dry, the centrifuge tube which acts as a receiver is shifted and then first 15 ml 1/10 n HCl is sucked through and afterwards 10 ml water. In this way all the glycocyamine is found in the filtrate while arginine and ammonia is in the eluate. Both fractions are evaporated in vacuo to a volume of 20 ml. Before the evaporation the eluate is made alkaline to phenolphthalein partly to precipitate the aluminum ions as hydroxide which is filtered off and partly to free the ammonia which then is distilled off. Half of the evaporated samples, 10 ml, is transferred to a 25 ml flask and the colour is produced according to MACPHERSON's modification (l. c.) of the Sagaguchi-reaction with the only difference that here is used 3 additions of urea and hypobromite to stabilize the colour. The readings are made in an electric photometer (REHBERG 1943) using a green filter (SCHOTT u. GENOSSEN V. G. 9). Besides the samples a pair of blanks are run. The blank has a rather high value and may vary a little from time to time depending mainly on the age of the chemicals being most stable when the hypobromide is some days old. The absolute concentrations are read on a standard curve (fig. 1) presenting the relation between the amount of arginine in mgs and the galvanometer readings — the blank. As the photometer scale is logarithmic, the straight course of the curve means that Lambert-Beers law is satisfied.

Control experiments in table 1 show that practically all glycocyamine added to urine is refound in the filtrate.

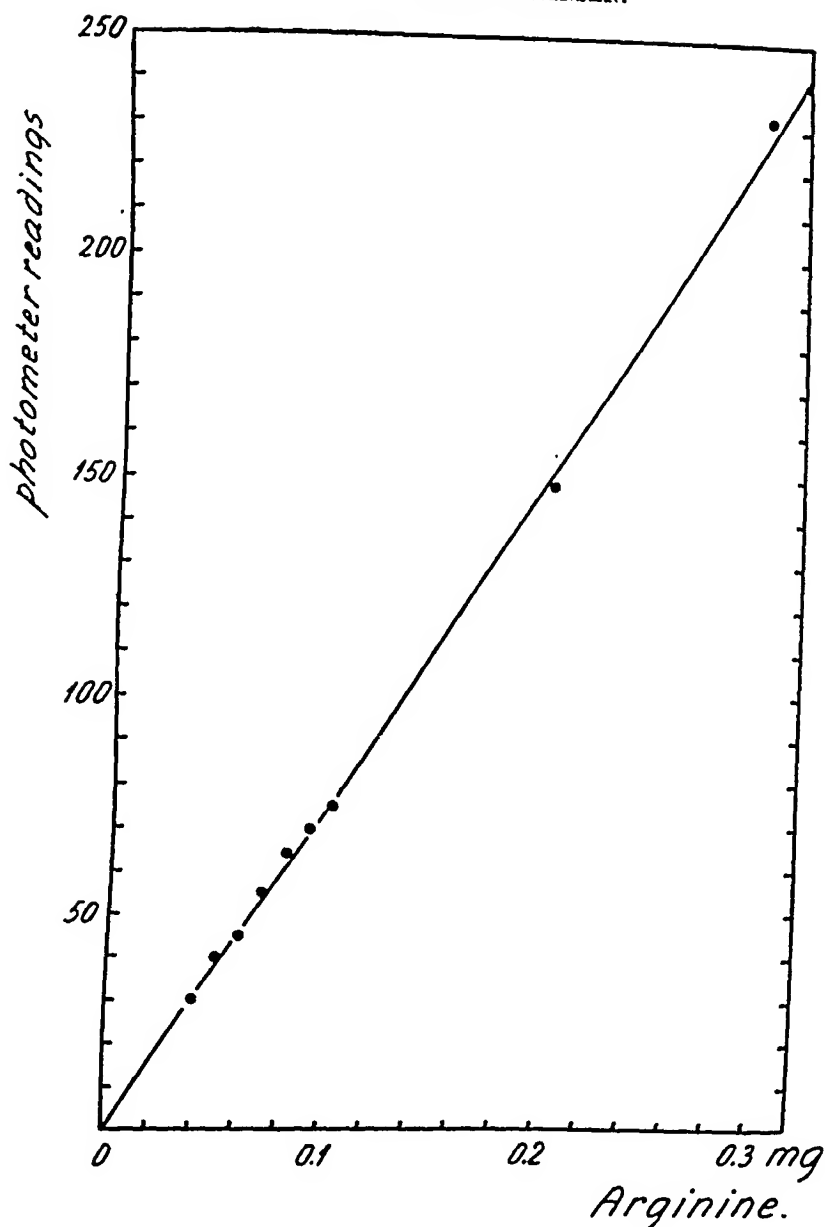


Fig. 1.

Table 2 shows likewise that nearly all arginine added to the urine is reformed in the eluate.

On serum the method can be used without any alterations. About 20 ml of blood taken from an arm-vein is collected in a centrifuge tube and immediately centrifuged; after a quarter of an hour the coagulum is loosened from the sides of the tube, and the

Table 1.

<i>Exp. I.</i>	2 ml urine	1 ml glycocyamine solution.	2 ml urine + 1 ml glycocyamine solution
Filtrate	155	450	600
Eluate	60		60
<i>Exp. II</i>			
Filtrate	80	450	536
Eluate	58		56

Table 2.

<i>Exp. I</i>	2 ml urine	1 ml arginine solution	2 ml urine + 1 ml arginine solution
Filtrate	132	45	130
Eluate	53		95
<i>Exp. II</i>	2 ml urine	2 ml arginine solution	2 ml urine + 2 ml arginine solution
Filtrate	153	88	155
Eluate	80		165

Table 3.

<i>Exp. I</i>	4 ml serum	1 ml arginine solution	4 ml serum + 1 ml arginine solution
Filtrate	0	80	3
Eluate	32		114
<i>Exp. II</i>	4 ml serum	2 ml arginine solution	4 ml serum + 2 ml arginine solution
Filtrate	0	155	2
Eluate	30		185

centrifuging is carried on until the coagulum has retracted. The serum is diluted with two volumes of water and 1 volume 20 % trichloroacetic acid is added. After standing an hour at room temperature a further volume of water is added, and the pre-

precipitate is filtered off. The trichloroacetic acid is extracted with ether for one hour in an extraction apparatus, and the filtrate is concentrated in vacuo to such a volume, that one ml corresponds to one ml of the original serum. 8 ml, neutral to lithmus, is made 70 % as regards ethanol, absorbed and treated in the same way as described under the urine analyses.

Table 3 shows some control experiments.

Judging from these figures it might be assumed that an adsorption should not be necessary, the filtrate having nearly the same extinction as the blank. But one of the persons used for the experiments had very high values for the filtrate, so adsorption could not be omitted and was performed in all cases.

Table 4 gives examples of the content of arginine in urine and serum from different persons who had not previously been fasting.

Table 4.

Arginine concentration mg %	
serum	urine
1.52	3.40
1.74	3.30
1.78	3.40
2.69	2.30
1.23	2.43

With a diuresis of 1.5 l per day about 50 mg arginine is thus excreted while about 2.00 g a day pass out into the glomerulus filtrate when the filtration is taken as 150 l. By far the most of the arginine is consequently either actively reabsorbed or diffuses passively back from the tubuli. Should a passive back diffusion be in question it was to be expected that the excretion of arginine would be increased at high diuresis. Experiments were made in which the persons had first obtained a low diuresis after thirsting and then had forced the diuresis up to a high value by drinking about 2 l water.

From table 5 which presents the results it is seen, that the excretion of arginine does not vary much from the low to the high diuresis; in the first two cases it is even lower at the high diuresis, so it may be assumed that arginine is actively reabsorbed. This reabsorption may be realized with a mechanism which either may

be specific to arginine or common to several amino acids. Should the latter be the case it might be supposed that the excretion of arginine would be increased after intake of a large amount of another amino acid.

Table 5.

diuresis ml/hour	arginine mg% in urine	mg arginine excreted/hour
27.3	3.50	0.955
560.0	0.15	0.840
<i>Exp. II</i>		
28.8	3.15	0.907
480.0	0.185	0.880
<i>Exp. III</i>		
29.6	2.91	0.861
736.0	0.145	1.063

Only one experiment of this kind has been made and the result is shown in table 6.

The person used for this experiment had been fasting for twelve hours. One hour before the intake of glycine the urine was voided and a blood sample of ca. 20 ml was taken from an arm-vein. 20 g glycine were given in 250 ml water and the urine was voided one hour later and again after 2 and 3 hours. A blood sample of 20 ml was taken 1½ hour after the glycine intake. Plasma and urine were analysed for arginine in the usual way. The glycine analyses were carried out after the method described by USSING (1945). In the case of the plasma only the total $\text{NH}_2\text{-N}$ was determined, partly because the procedure with the ten times absorption on coal claims a relatively large amount of blood, and partly because it was not absolutely necessary as the corresponding value for glycine of the first sample could be deduced from the tables of USSING (l. c.) and for the second sample the whole difference in $\text{NH}_2\text{-N}$ between the first and the second was calculated as glycine. The analyses of the urine on the other hand were performed exactly according to the method mentioned. The rate of filtration was not determined; in the calculations the value of inulin clearance (120 ml/min.) has been used in both cases. This is not quite

Table 6.

periode	diurese ml/hour	glycine							
		plasma		urine		excreted mg/hour	filtered mg/hour	reabs. mg/h.	reabs. %
		total NH ₂ -N mg %	conc. mg %	total NH ₂ -N mg %	conc. mg %				
Last hour before gly- cine intake	25	4.96	6.54	15.15	26.9	6.75	470.88	464.13	98.5 %
1. hour af- ter glycine intake	22	—	—	42.00	146.0	32.12	—	—	—
2. hours ..	24.5	6.98	17.34	118.50	515.0	126.18	1 248.48	1 122.30	89.8 %
3. hours ..	34.5	—	—	145.00	670.0	231.15	—	—	—
arginine									
last h.	25	4.96	1.78	15.15	3.42	0.86	111.2	110.34	99.2 %
1. h.	22	—	—	42.00	3.20	0.71	—	—	—
2 h.	24.5	6.98	1.71	118.50	2.40	0.59	123.12	122.53	99.5 %
3. h.	34.5	—	—	145.00	2.70	0.93	—	—	—

correct as PITTS (1944) has shown that the filtration rate increases with the amino acid intake. He finds in his experiments on dogs that the filtration increases from 60—80 ml/minute when the corresponding total NH₂-N in plasma increases from 4—20 mg%. The variation in this experiment however in plasma total NH₂-N is from 4.96—6.98, so the error in using the same value for the filtration cannot be very important.

As far as anything can be concluded from one experiment, this seems to suggest that the reabsorption of arginine is quite unaffected by glycine and that apparently they are not reabsorbed by the same mechanism. PITTS (1944) assumes a mechanism common to all amino acids preferring glycine to alanine and in decreasing order glutamic acid and arginine. This does not quite agree with USSING (l. c.) whose experiments seem to indicate that glycine is not reabsorbed by the same mechanism as the other amino acids examined (histidine, tyrosine). If, however, the mechanism is a common one, the affinity order set up by PITTS might perhaps

be reversed at low concentrations, arginine in that case being more effectively reabsorbed than glycine.

Summary.

A method is described for determining arginine in urine and serum colorimetrically after adsorption in 70 % alcoholic solution on an alkaline column of Al_2O_3 and subsequent elution with 1/10 N HCl.

A few experiments regarding the reabsorption of arginine in the renal tubules are reported.

The author wishes to express her sincere thanks to the head of the institute Professor P. BRANDT REHBERG for placing all the laboratory facilities at her disposal.

My thanks are also due to Dr. USSING for his great helpfulness and advice.

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Renal Excretion of Glycerol.

By

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HOLST (1943) investigated the excretion of glycerol in the kidneys of narcotized rabbits during continuous intravenous administration of glycerol. The main purpose of his experiments was to ascertain whether poisoning with phloridzin has an effect on the glycerol excretion corresponding to its effect on the excretion of glucose. HOLST's results show that such is not the case.

As glycerol is phosphorylated with great intensity by the tissue of the renal cortex *in vitro*, and as this phosphorylation is inhibited by phloridzin, HOLST's findings meant a very considerable weakening of the principal arguments that can be advanced in support of the theory that phosphorylation constitutes a stage in active reabsorption of glucose in the kidneys. These arguments are, as we know, to the effect that glucose is phosphorylated with great intensity by renal cortex tissue *in vitro*, that this phosphorylation is specifically inhibited by phloridzin and that phloridzin checks the reabsorption of glucose in the kidneys.

The above-mentioned arguments in favour of the theory of phosphorylation as a stage in the active reabsorption of glucose can after HOLST's observation be applied only if it is at the same time assumed that the excretion of glycerol in the kidneys differs fundamentally from the glucose excretion.

HOLST's investigations on rabbits indicate that such is the case, since he found that the excretion percentage E (the percentage of the glycerol filtered through the glomeruli that is excreted in the urine) is constant in one and the same animal, irrespective of the glycerol concentration in plasma and of the excretion of water.

E varied, however, greatly from animal to animal, from a small percentage up to 60 per cent. HOLST was unable to ascertain whether glycerol is a threshold substance or not, but he drew the conclusion that, if it is a threshold substance, then the threshold is very low, under 10 mg per 100 ml.

The present work embraces a re-investigation of HOLST's findings, with use of human subjects of experiment, partly because with the methods employed the determination of glycerol can be more easily effected in human urine than in rabbit urine, as also HOLST has noted, and partly in order to avoid the use of narcotics.

Methods.

HOLST's method for determination of glycerol in blood and urine is based upon a reaction between acrolein and anthron (BALLY and SCHOLL 1911), whereby is formed benzanthron, which in sulphuric acid solution has an orange-red colour and gives olive-green fluorescence. The method has been employed by SCHÜTZ (1938) for colorimetric determination of glycerol in aqueous extract. HOLST employed the same colorimetric method after having obtained precipitation of protein and other disturbing substances by means of basic acetate of lead, zinc sulphate and sodium hydroxide. In case of urine this precipitation was effected after previous treatment with activated charcoal. HOLST carried out the colour test in a quantity of liquid corresponding to 0.1 ml of plasma or blood and in case of urine corresponding to 0.1 ml of a urine filtrate obtained after the excreted urine had been diluted and treated with charcoal. An ordinary DUBOSQ colorimeter was used for the analyses. LUNDGAARD (1946), when applying HOLST's method for determination of glycerol, used HAVEMANN's photo-electric colorimeter to determine the absorption of light. In the present investigation BECKMAN's spectrophotometer was employed, whereby it has been possible to measure the absorption of monochromatic light. In this way greater specificity was attained than by the above-mentioned apparatuses, as the colour developed has a very characteristic absorption spectrum (Fig. 1).

The mode of procedure in the present investigation is mainly the same as was adopted by HOLST,¹ but a few alterations must be mentioned. HOLST precipitated the protein in 0.2 ml of blood or plasma and made the colour test in a quantity of liquid corresponding to one half thereof, but in our analyses of blood or plasma to which glycerol

¹ LUNDGAARD (1946) mentions a discrepancy between his and HOLST's results which had the character of a systematic error and which could best be explained as being due to the employment of a different standard by LUNDGAARD than by HOLST. The discrepancy was due to LUNDGAARD having supposed that the tables to which HOLST refers, showing the density of the glycerol solutions, gave the glycerol content per 100 ml, whereas they gave the content per 100 g.

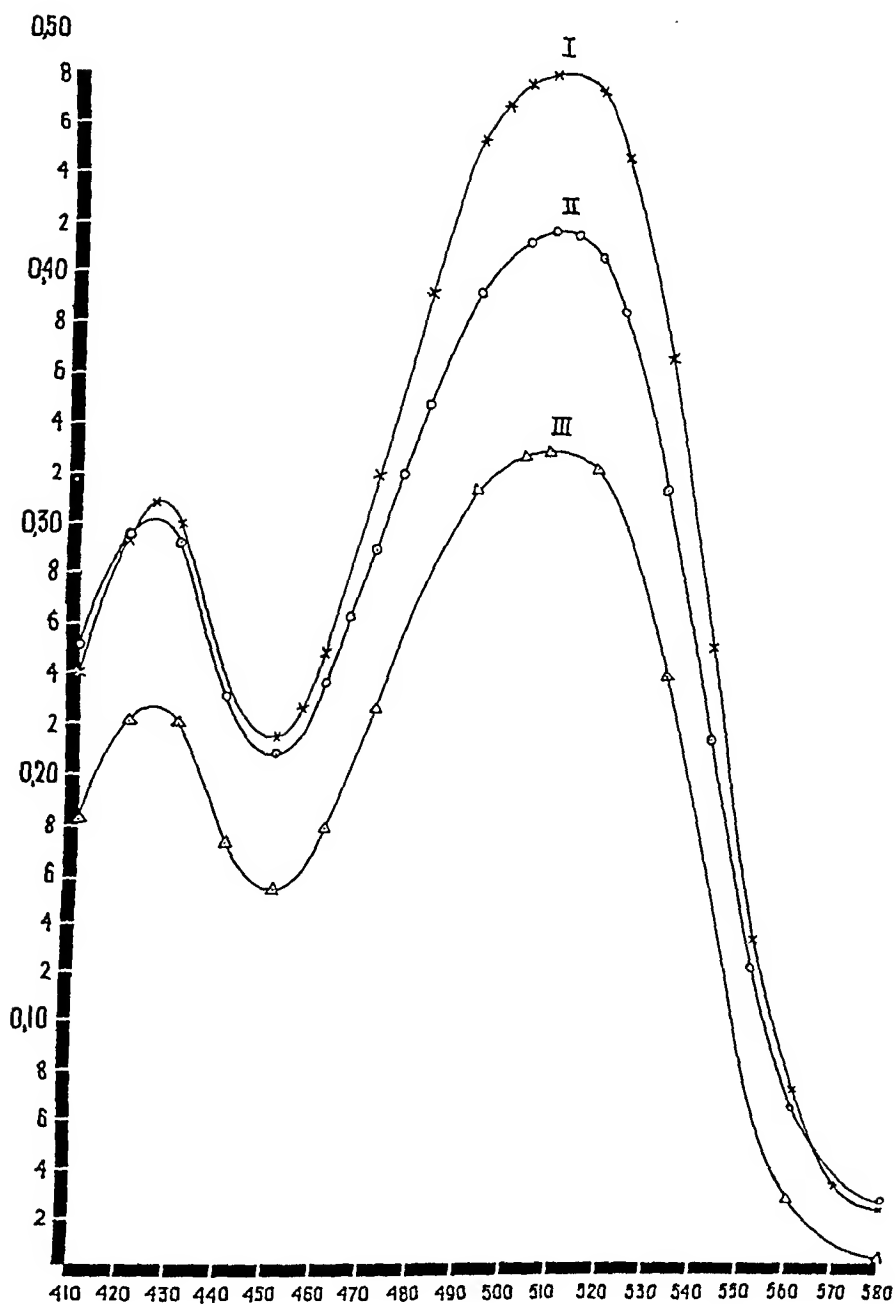


Fig. 1. Absorption spectra of benzanthrone from.

I. Blood after administration of glycerol. Dil.: 1/20.

II. Urine , , , Dil.: 1/50.

III. Pure glycerol solution 1.05 mg%.

Ordinate: Extinction, $\log 10/I$ for 1 cm.

Abscissa: Wavelength in $m\mu$.

Table 1.

Dissolving medium	Size of sample	Glycerol concentration in sample	Blank value of blood serum and urine	Result of analysis	Analysis minus blank	Recovery in per cent of given concentration	Average recovery
Water		1.87		1.82		98.3	98.9
						98.3	
		1.36		1.33		97.8	
		1.11		1.09		98.2	
		1.36		1.31		96.3	
		0.57		0.59		103.5	
		2.99		2.98		99.7	
		3.98		4.04		101.6	
		3.16		3.10		98.4	
		3.16		3.12		98.7	
		2.10		2.09		99.3	
		4.20		4.16		99.0	
		2.10		2.08		99.0	
		4.20		4.08		97.0	
		4.20		4.14		98.5	
Urine		1.87	0.07	1.82	1.75	93.6	98.4
		4.98	0.05	5.02	4.97	99.8	
		2.49	0.06	2.51	2.45	98.4	
		1.36	0.06	1.35	1.29	95.5	
		4.98	0.05	5.03	4.98	100.0	
		2.49	0.06	2.55	2.49	100.0	
		1.36	0.06	1.36	1.30	95.6	
		1.36	0.08	1.40	1.32	97.1	
		0.57	0.09	0.69	0.60	105.3	
Blood	0.2	2.99	0.11	2.77	2.66	89.1	
	0.2	2.99	0.11	2.99	2.88	96.5	
	0.2	3.98	0.28	3.93	3.65	91.7	
Serum	0.2	2.10	0.19	2.00	1.81	86.2	91.3
	0.2	4.20	0.12	4.04	3.92	93.4	
	0.2	2.10	0.12	2.63	1.91	91.0	
Serum	0.1	4.20	0.06	4.26	4.20	100.0	97.3
	0.1	2.10	0.06	2.06	2.00	95.3	
	0.1	2.10	0.06	2.11	2.05	97.6	
	0.1	1.05	0.06	1.07	1.01	96.3	

had been added in known concentration it was found that the highest percentage of the added glycerol was recovered when only 0.1 ml was employed in the precipitation procedure (Table 1). The table records the results of a series of recovery experiments.

The quantity of anthron added to the sample containing glycerol is small (4 ml of a 10 mg % w/v solution), which is especially necessary in case of ordinary colorimetric procedure, as the anthron has itself an intense colour. Although the quantity of anthron added can

at most react only with the glycerol contained in 2 ml of a 9.6 mg % solution, as pointed out by HOLST, he made his analyses in samples of up to 15 mg% solutions. The accuracy of the readings at this high concentration of glycerol will thereby be necessarily somewhat reduced.

In the present work the maximum concentration of glycerol in the corresponding solution taken was fixed at 5 mg % w/v. Within this range the relation between the extinction and the glycerol concentration in the sample is approximately linear for the wave-length employed: 500 m μ . In order to gain greater exactitude, however, the determination of the concentration was effected by means of a standard curve. It has occasionally been necessary to correct this standard curve, especially when one or more of the reagents were changed.

The temperature and time of heating were the same as adopted by HOLST. In comparative investigations it was found that small variations in temperature and time of heating ($\pm 10^\circ$ or ± 3 min.) led to very slight variations in the colour obtained and had no influence when the extinction was, as is usual, read off against the blank (water + reagents that had undergone exactly the same treatment as the sample). The colour obtained was very durable at room temperature. After standing for 18 hours no change in the extinction could be noted.

Apart from the small alterations mentioned above, the mode of procedure in the analysis of blood and plasma was the same as described by HOLST. As regards the urine, the concentration of glycerol was first reduced by dilution to below 10 mg % w/v. Thereupon 2 ml of the diluted urine were pipetted into a centrifuge tube, already containing 0.7 ml of water. The subsequent procedure was exactly the same as for blood or plasma.

Experimental Investigations.

The experiments included a determination of the glycerol excretion in urine with varying concentration of glycerol in the blood and varying excretion of water. Altogether 17 experiments were carried out on 3 different persons, all of whom were adult men. Subject A, weight 74 kg: 9 experiments, Subject B, weight 75 kg: 6 experiments, and Subject C, weight 64 kg: 2 experiments. The experiments were always made in the morning at the same time. The persons were usually fasting and in some cases they had not drunk anything since the previous day. After a sample of blood (capillary blood with addition of heparin) and a sample of urine have been taken, a dose of glycerol dissolved in more or less water is given in order to raise the glycerol content in the blood to the desired height. After the lapse of an hour (pre-period) the bladder is emptied, a new sample of blood is taken and a small dose of glycerol is given at intervals of 10 min. in the following two periods of 1 hour for the purpose of maintaining an approximately constant concentration of glycerol

Table 2.

Exp. No	Period	Average blood glycerol in period mg% w/v	E	Average diuresis during period ml/min	Glycerol concentration in urine mg% w/v	Excretion of glycerol in urine mg/min.
15	4	3.5	4.4	0.46	41.7	0.2
15	3	4.4	4.1	0.46	48.4	0.2
15	1	5.4	3.6	0.46	52.0	0.2
4	2	5.4	12.6	0.63	126.0	0.9
15	2	6.9	3.4	0.43	52.2	0.2
16	2	¹ 7.1	2.1	0.42	43.8	0.2
3	1	7.2	1.8	0.55	28.8	0.2
8	1	7.5	9.3	7.54	11.6	0.9
4	1	7.8	8.6	0.73	113.5	0.8
9	1	10.7	9.2	0.63	195.0	1.2
16	1	¹ 11.1	3.6	0.39	127.1	0.5
9	2	11.5	8.0	1.10	176.0	1.9
6	1	13.7	18.5	1.08	292.0	3.2
6	2	14.7	17.6	1.27	255.0	3.2
8	2	15.7	29.3	8.71	66.0	5.7
3	2	19.3	34.5	0.73	1130.0	8.3
11	2	31.0	54.2			21.0
11	1	34.1	44.2	1.28	1878.0	18.8
3	3	34.5	49.6	1.00	2135.0	21.4
7	2	40.9	44.8	8.96	255.0	22.9
7	1	41.9	40.8	7.10	302.0	21.4
5	2	47.6	36.0	1.75	1225.0	21.5
13	2	48.4	38.3	0.73	3110.0	23.1
1	2	52.1	48.7	1.08	2920.0	31.7
5	1	52.1	39.0	1.90	1340.0	25.4
13	1	53.3	35.4	0.63	3740.0	23.6
1	1	54.7	56.4	1.26	3060.0	38.5
2	2	55.5	48.3	1.07	3140.0	33.5
10	1	60.5	35.5	0.91	2950.0	26.8
10	2	61.1	37.4	0.97	2958.0	28.6
2	1	61.9	42.0	1.10	2950.0	32.5
12	2	89.3	51.0	1.61	3470.0	55.9
14	2	91.3	48.0	1.91	2860.0	54.7
12	1	96.7	47.4	1.53	3735.0	57.3
14	1	102.1	39.1	4.05	1233.0	49.9

in the blood (LUNDGAARD 1946). Every 20th minute a blood sample is taken and the bladder is emptied after one hour and after two hours. The amount of glycerol that must be added in order to maintain a constant concentration of glycerol in the blood — corresponding to the elimination — will, with low and medium-high glycerol contents in the blood, be the same as stated by HOLST, namely, 2.5 mg per kg of body-weight per min. With very high glycerol concentrations in the blood, however, more than this quantity will be required to keep the content of glycerol constant, which fact can, at any rate partly, be explained by the increased excretion of glycerol in the urine at high concentrations of glycerol in the blood.

¹ Plasma glycerol.

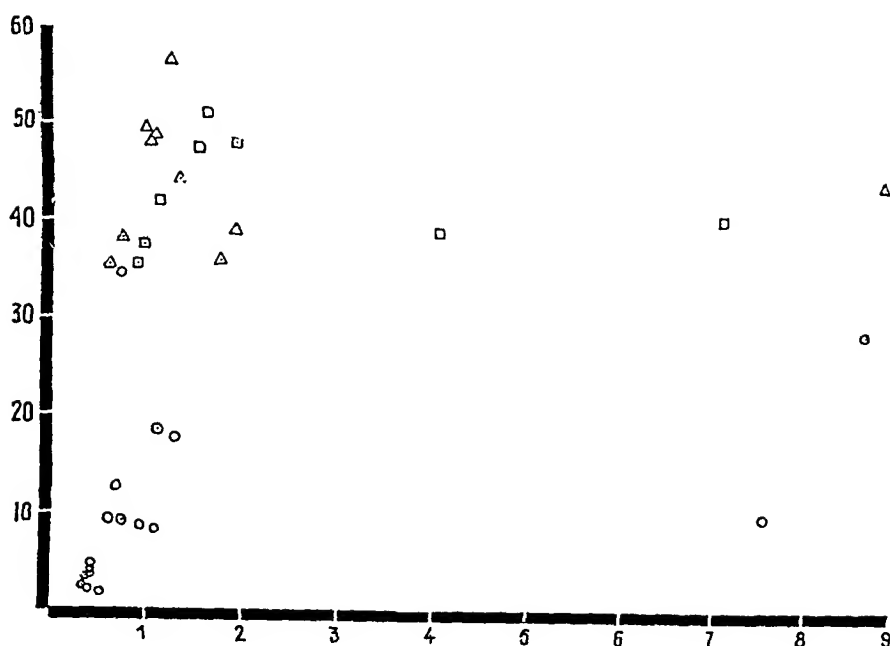


Fig. 2. E. in relation to diuresis.

Ordinate: E percentage.

Abscissa: Diuresis in ml per min.

○: Blood glycerol 0 — 19.8 mg %.

△: , , 31.0—55.5 mg %.

□: , , 60.5—102.1 mg %.

The results of the experiments are given in Table 2, where the periods are arranged according to the average concentration of glycerol in the period. The percentage of excretion is reckoned on the basis of an assumed filtration of 125 ml per minute. In these orientatory tests determination of filtration was omitted. As the tests were carried on the same subjects under uniform conditions and in the course of a short period, it is justifiable to suppose that any variations occurring in the filtration have been insignificant.

It appears from the results of the experiments that the excretion percentage E rises with increasing glycerol concentration in the blood, up to a content of about 20—30 mg %, and reaches a value of 54.2 per cent at a blood glycerol level of 31 mg %. If the glycerol concentration in the blood increases from about 30 mg % to near 100 mg %, no tendency to continued rise in the excretion percentage can be noted, seeing that this percentage in the different periods varies between 35 and 56 per cent in the three subjects of experiment.

No certain interdependence between the diuresis and the percentage of excretion can from these experiments be seen to exist (Fig. 2). Especially it may be noted that profuse diuresis of .7—8 ml per minute leads to no increase of E. Whether very small diuresis leads to reduction of E cannot be seen from these experiments, as diureses below 0.5 ml per minute were noted only in experiments where the glycerol concentration was very low.

Discussion and Supplementary Investigations.

So long as we regard experiments with glycerol concentrations of more than 30 mg% in the blood as being in accordance with HOLST's observations in case of narcotized rabbits, the conclusion to be drawn from the above-recorded experiments is that E is constant in the same individual and is independent of the glycerol concentration in the blood and of the excretion of water. With low concentrations of glycerol, less than 20 mg%, it has, on the contrary, been found that E decreases with falling glycerol concentration in the blood. HOLST has in his experiments very few experimental periods in which the glycerol concentration was low, for example, only two with concentration under 10 mg%, and in these cases the animals were phloridzin-poisoned. In these two experiments he finds low values for E, but believes he can explain them by assuming the constant presence of a blank value in the blood analysis whereby the glycerol concentration has been reckoned too high and the E value therefore too low. At the low glycerol concentrations such an error will have most effect on the percentage, and on the basis of this reasoning HOLST maintains his above-mentioned opinion.

As HOLST pointed out, glycerol differs considerably from glucose in its mode of excretion in the kidneys. Two circumstances may be supposed to contribute to render the manner of excretion of glycerol in the kidneys different from that of glucose. Firstly: the great diffusibility of glycerol. Secondly: the small T_m which the glycerol may naturally be supposed to have. It is reasonable to suppose that the T_m of an actively absorbed substance is to some degree adjusted according to the quantity of the substance filtered under normal circumstances.

The peculiarity that for plasma values above 20—30 mg % the excretion percentage (E) of the glycerol is constant and in-

dependent of the diuresis may possibly be explained in a manner which has been suggested by LUNDGAARD (personal communication). The easily diffusible glycerol is supposed to diffuse back into the proximal part of the tubuli together with the quantity of water there reabsorbed. In the distal part of the tubuli, where the facultative reabsorption of water takes place and the diuresis is regulated, it must be supposed that no diffusion of glycerol takes place, and consequently the glycerol concentration in the urine will increase. The E of the glycerol will, however, remain constant, since this factor is dependent only on the extent in which the filtered water with its glycerol content is reabsorbed in the proximal part of the tubuli.

In order to explain why E in case of glycerol concentrations of less than 20—30 mg% decreases with decreasing blood glycerol content it is necessary to assume that an active reabsorption of glycerol takes place together with the passive diffusion. The actively reabsorbed quantity (T_m) is small and, as long as the glycerol concentration in the blood is high, it will be insignificant as compared with the quantity of glycerol which passively diffuses back. Therefore at high blood glycerol values the excretion percentage of the glycerol will be only slightly affected by the active reabsorption. It is a different matter when the concentration of glycerol in the blood is small. With falling concentration of glycerol in the blood the actively reabsorbed glycerol will constitute an increasing proportion of the filtered quantity. In other words: E will decrease.

It is obvious that when the glycerol concentration in blood and urine is small a methodical error or the presence of a blank value will have more effect than when the content of glycerol is larger. It is therefore necessary to examine whether there are present in the blood and urine other substances than glycerol which under the given conditions may produce a colour that can be mistaken for the colour produced by glycerol. On investigation of blood and urine samples there will always be found a certain colour corresponding to a particular concentration of glycerol. In 10 such samples of blood were found "glycerol" values of from 1.6 to 3.6 mg% and in corresponding urine samples values of from 5 to 37 mg%. The larger range of variation for the urine must be regarded in connection with the great variation in the concentration of the urine in the different experiments. On examination of the absorption spectrum of the colours produced with anthron

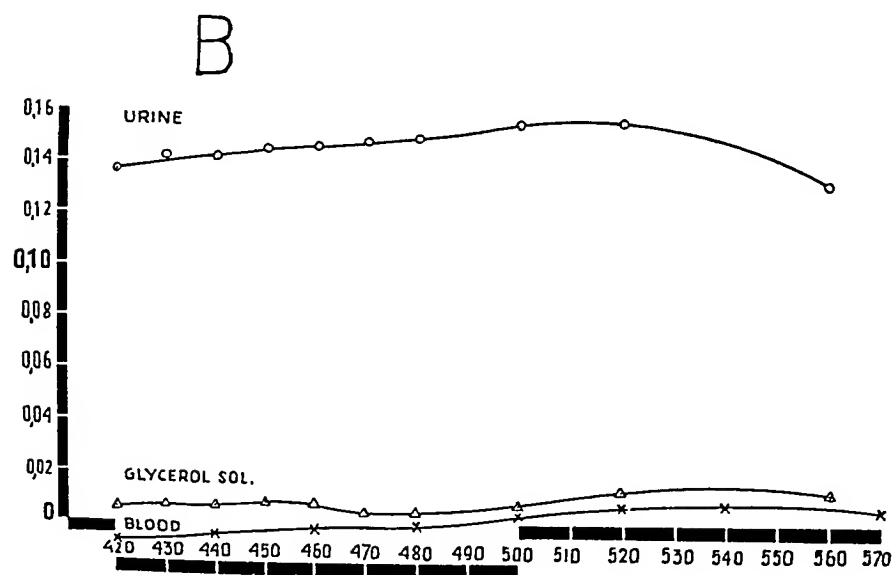
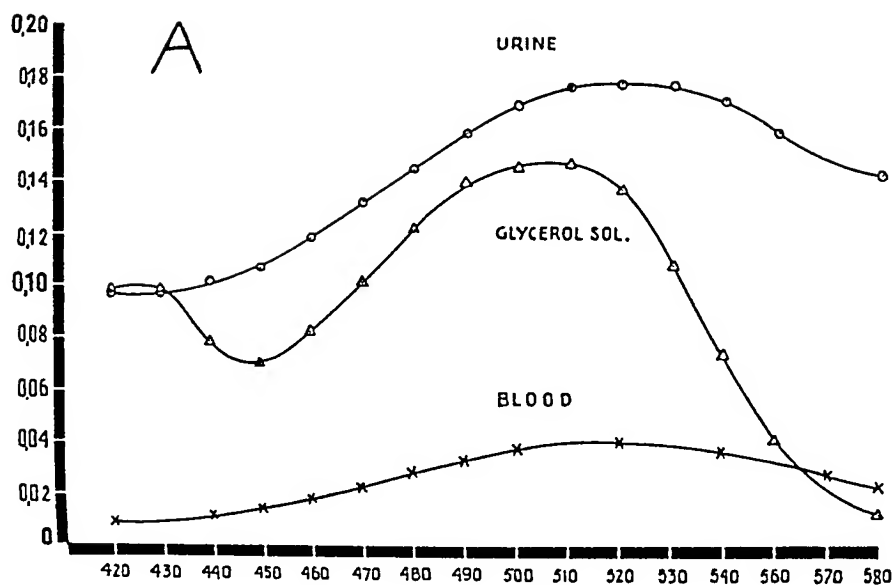


Fig. 3. A. Absorption spectra of the colour produced with anthron in a pure glycerol solution and in samples of urine and blood taken before administration of glycerol.
 B. Absorption spectra of colour produced in the same 3 fluids after splitting of glycerol with periodic acid.
 Ordinate: Extinction, $\log I_0/I$ for 1 cm.
 Abscissa: Wavelength in $m\mu$.

before administration of glycerol it is found that both for blood and urine the absorption spectrum differs in some degree from that obtained from a pure glycerol solution and likewise from blood and urine undoubtedly containing glycerol (Figs. 1 and 3). As regards the urine, it can be concluded from the appearance of the absorption spectrum that other coloured compounds than benzanthron contribute, at least in part, to the light absorption observed. With respect to the blood the matter is less clear. The characteristic features in the absorption spectrum of the colour produced in blood-filtrate are somewhat blurred, but the concentration of the coloured substance is here so small that this circumstance loses much of its weight:

That there is an essential difference between the absorption spectrum of the coloured substance produced in filtrates from urine and from blood before administration of glycerol has, however, been proved by the power of periodic acid to decompose glycerol. FLEURY and LANGE (1933), FATOME (1935) and MALAPRADE (1937) have shown in a series of works that periodic acid can split organic substances in which two alcoholic groups are present side by side. Through the splitting up of glycerol are produced formic acid and formaldehyde, the periodic acid being reduced to iodic acid. In the present investigation the fluid obtained after urine or blood had undergone the precipitation process was treated with a small quantity of periodic acid, 0.02 ml of an M/10 periodic acid solution, added to the total centrifugate obtained after the second centrifugation. After standing for 15 minutes at room temperature 2 ml of this centrifugate are pipetted off for the execution of the previously described colour test. The blank is treated in the same manner. It has been found that the substance in the blood filtrate that produces the colour in presence of anthron practically entirely disappears on treatment with periodic acid, whereas from the urine only a small part of the substance is lost through such treatment. Therefore the main part of the substances causing the colour development in a urine sample before administration of glycerol does not consist of glycerol. As regards blood, the combination of the two properties of being able to produce this colour in presence of anthron and of being decomposable by periodic acid strongly indicates that the substance present is glycerol. It is assumed that, besides protein, lipoids and glucose, also glycerophosphates have been removed by the precipitation procedure employed (PAULSEN 1941).

Thanks to this property of periodic acid, it is possible to distinguish between administered glycerol, "endogenous glycerol" and a group of substances differing from glycerol, but which under the given conditions develop a certain colour within the range of light absorption of benzanthron. In some of the experiments, where the glycerol content was very small, E was calculated after exclusion of the colour-producing substances other than glycerol (Table 3). From the table it is seen that the E value calculated after deduction of colour producing substances which are not glycerol is still lower than that found on calculation in the usual manner.

Table 3.

Experiment No	Period	E calculated from total amount of colour-giving substances in blood and urine	E calculated after subtraction of colour-giving substances other than glycerol
15	I	3.6	2.1
15	II	2.7	1.6
15	III	4.1	1.8
15	III	4.4	2.6
16	I	3.6	2.7
16	II	2.1	0.8

As already mentioned, the concentration of "glycerol" in blood and urine before administration of glycerol was determined in a series of experiments. In some of these cases periodic acid was also employed, whereupon it was found that the concentration of "endogenous" glycerol in the urine (colour-producing substances, decomposable by periodic acid) was very small, not differing much from the concentration of corresponding substances in the blood (Table 4). In one experiment the excretion of "endogenous" glycerol during copious diuresis was examined.

Table 4.

	Concentration of glycerol in blood mg% w/v	Concentration of glycerol in urine mg% w/v
Experiment No 15	3.0	6.4
" " 16	3.0	0
" " 17		2.6
" " 18	4.4	0.1

45 minutes after Subject A had drunk 1 litre of water the bladder was emptied and a blood sample was taken from a vein in the arm. After the lapse of 30 minutes the bladder was again emptied and a new sample of blood was taken. The average diuresis had been 7.2 ml per minute. The concentration in the blood of substances which displayed colour on treatment with anthron was, expressed as glycerol, 4.0 and 4.8 mg% respectively in the two samples. (The subject was not fasting.) The concentration of such substances in the urine was 1.03 mg%. After treatment with periodic acid the blood filtrate was practically free from substances which showed colour when treated with anthron, whereas the urine still contained such substances in a concentration of 0.93 mg %. Herefrom it follows that when the content of glycerol or of substances closely related to glycerol was 4.4 mg% in the blood, the content of such substances in the urine was only 0.1 mg%, which gives a concentration index of 0.02. E could be calculated to be 0.1 per cent.

These investigations show that the substance designated "endogenous" glycerol is a threshold substance. Whether supplied glycerol also appears as a threshold substance could not be ascertained in the same manner, since the concentration index in all the experimental periods lay above 1. However, we find support for the assumption that exogenous glycerol is a threshold substance on comparing the quantity of glycerol excreted per minute in the urine with the concentration of glycerol in the blood (Fig. 4). The diagram shows that the excretion of glycerol (actually the total quantity of colour-giving substances), which is very small at the lowest concentrations in the blood, does not show any rapid increase until the concentration of glycerol in the blood has reached to more than 8—10 mg %. It seems natural to suppose that the threshold for the glycerol lies within this range of concentration.

As already mentioned, determination of glomerulus filtration was not carried out in these orientatory experiments, but the calculation of E was made on the basis of an assumed formation of 125 ml of glomerulus filtrate per minute. In most of the experiments, however, the concentration of glycerol was determined in whole blood instead of in plasma. In view of the great diffusibility of glycerol and its easy solubility in water there is reason to believe that the concentration of glycerol in blood corpuscles and plasma is determined by the content of water in these constituents of the blood. The glycerol concentration in plasma should there-

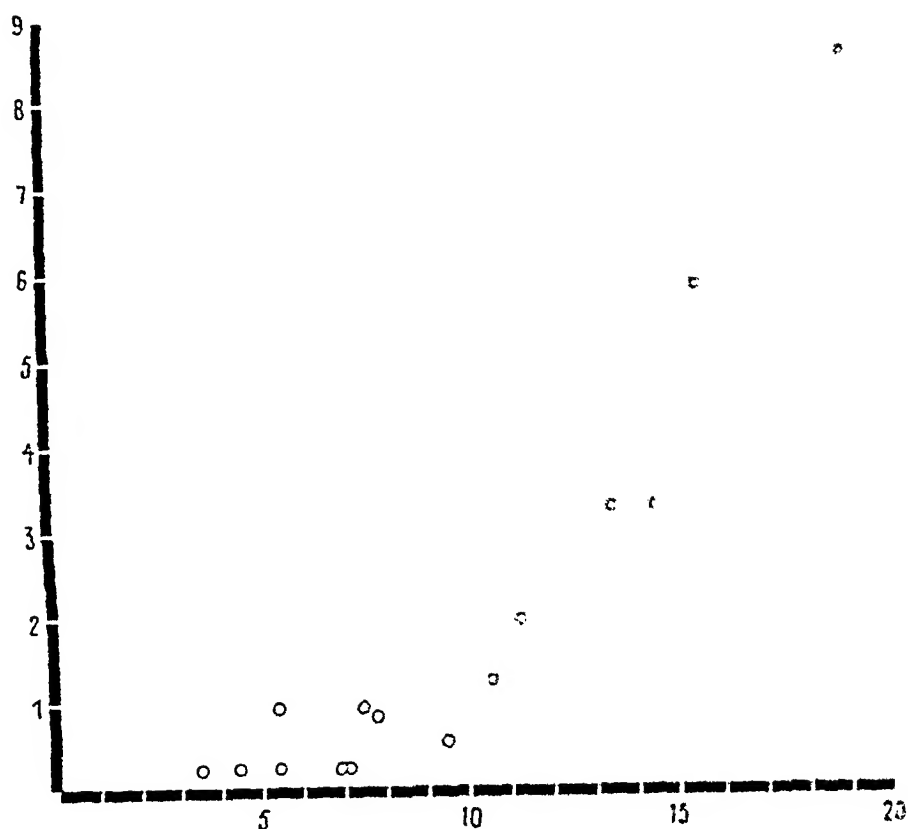


Fig. 4. Excretion of glycerol in urine in relation to concentration of glycerol in blood.

Ordinate: Mg glycerol excreted in urine per min.
 Abscissa: Concentration of glycerol in blood.

fore be higher than in whole blood, which assumption has also been confirmed by simultaneous analyses of whole blood and plasma (Table 5). It has been found that the average concentration in plasma lies 14 per cent above the value noted for whole blood. Consequently, the calculated excretion percentages should be correspondingly lower than those shown in the different tables. The interdependence found to exist between the glycerol content in the blood and the excretion percentage will, however, not be affected by such correction.

If the glycerol, as my results seem to show, is a threshold substance with very low T_m , the excretion of which, however, at only fairly high plasma-glycerol concentrations is completely governed by a passive retrodiffusion, then the findings recorded by HOLST

Table 5.

Sample No	1	2	3	4	5	6	7	8	9
Glycerol concentration in blood mg ^o / ₁₀₀ w/v	82.0	59.2	54.8	7.4	38.0	52.4	49.6	52.4	41.6
Glycerol concentration in corresponding plasma mg% w/v	90.8	70.8	62.8	8.4	42.4	58.4	54.8	61.2	48.0
Glycerol concentration, blood/Glycerol concentration, plasma	0.90	0.84	0.87	0.88	0.90	0.90	0.86	0.91	0.86

do not represent any weakening of the arguments in favour of the "phosphorylation theory".

That poisoning with phloridzin does not affect the glycerol excretion in the same manner as it affects the glucose excretion is due to the fact that the glycerol excretion at only fairly high concentrations of plasma-glycerol is governed by the passive retrodiffusion, so that a possible inhibition of the weak active reabsorption will not become distinctly evident.

The active reabsorption of glycerol may very well be imagined to involve a phosphorylation, and the power of the renal tissue to phosphorylate glycerol can therefore be regarded as having connection with the active reabsorption. It may be said, however, that the intensity with which glycerol is phosphorylated by the tissue of the renal cortex harmonizes badly with the low T_m found for glycerol. But conclusions respecting the rapidity of enzymatic processes in the intact tissue based upon the rapidity observed in extracts must always be drawn with all possible reservation.

Summary.

Orientatory investigations were made respecting the renal excretion of glycerol in man.

A method for the splitting of glycerol in biological samples by means of periodic acid is described. The method permits of distinguishing between glycerol and attendant substances which give colour reactions similar to those produced by glycerol.

At plasma-glycerol concentrations above 30 mg% the excre-

tion percentage has, in accordance with HOLST, been found to be independent of the concentration in plasma and of diuresis.

At lower plasma-glycerol concentrations the excretion percentage decreases with decreasing concentration in plasma.

These findings respecting the excretion are explained by assuming that glycerol is a threshold substance with low T_m (concentration threshold about 10 mg%), the excretion of which at higher concentrations of glycerol in plasma is, however, completely governed by a passive retrodiffusion of glycerol in the proximal tubuli.

Through this conception of the glycerol excretion HOLST's findings can be brought into harmony with the theory of phosphorylation as part of the process of active tubular reabsorption.

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Determination of Benzoyl-Glycuronic Acid in Urine.

By

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Benzoic acid, administered to man and animals (rat, rabbit, dog, swine, sheep), is excreted in the urine bound either to glycoll as hippuric acid (WÖHLER 1824) or to glycuronic acid as 1-benzoyl-glycuronic acid (MAGNUS-LEVY 1907, NEUBERG 1924, QUICK 1926—31, PRYDE and WILLIAMS 1936, GOEBEL 1937). Free benzoic acid is apparently not excreted in the urine. NEUBERG (1924) showed that this did not happen in man even if large doses of benzoic acid were administered (10 to 15 gm. in single doses). In rabbits GRIFFITH (1926) found that the excretion of free benzoic acid after administration of benzoate in amounts up to 1 gm./kg. was none, or anyhow inconsiderable. In experiments on rats, to which benzoate was given mixed up with the food, GRIFFITH (1928) found that practically all the benzoic acid was excreted as bound even if the amount of benzoic acid administered was so large as to prevent the growth of the animals. Earlier experiments, where varying amounts of free benzoic acid have been demonstrated in the urine, must be interpreted in another way; the presence of benzoyl-glycuronic acid may have been overlooked, or the experiments must have been performed with such a method that benzoyl-glycuronic acid must have been broken down to form free benzoic acid.

The occurrence of benzoyl-glycuronic acid is thus proved from a qualitative point of view, but the quantitative details and the

mechanism of detoxification are far from settled. This is due primarily to our want of a specific and sufficiently exact method of determining benzoyl-glycuronic acid.

The methods hitherto used for determinations of benzoyl-glycuronic acid follow one of the lines summarized below.

1. Determination of the glycuronic acid bound in the complex compound.

a. By means of a colorimetric reaction, based on the one described by TOLLENS (1908) between glycuronic acid and naphthoresorcine when heated with hydrochloric acid. This reaction is not specific for glycuronic acid. MOZOLOWSKI (1940) showed that urine often contains substances which disturb this reaction. Urine further contains glycuronic acid bound to other compounds than benzoic acid. WAGREICH, ABRAMS and HARROW (1940) thus detected a considerable excretion of glycuronic acid in the 24-hour urine in experimental subjects to whom no benzoic acid had been administered and who consequently excreted only traces of benzoyl-glycuronic acid. Their mean value from 38 determinations, 508 mg. of glycuronic, ranging from 300 to 859 mg., is best interpreted as an illustration of the inadequate specificity. All methods based on determinations of the glycuronic acid-component are thus non-specific.

b. Through determination of the reducing power of the urine: with Benedict's reagent (CSONKA 1924), with Shaffer-Hartmann's reagent (QUICK 1926). As non-diabetic urine, too, contains other reducing substances than glycuronic acid, methods based on a determination of the reducing power of the urine must be considered still less specific.

2. Determination of the benzoic acid in the compound as the difference between total benzoic acid and the benzoic acid bound in hippuric acid (NEUBERG 1924); total benzoic acid according to KINGSBURY and SWANSON (1921), hippuric acid according to SNAPPER and LAQUEUR (1924), and QUICK (1926); total benzoic acid according to KINGSBURY and SWANSON, hippuric acid according to QUICK (1926). It is true that this method of determining benzoyl-glycuronic acid is specific — under the assumption that no free benzoic acid is excreted — but it can be applied to certain species only. In most cases — *i. a.* in man — the amount of benzoyl-glycuronic acid is small in relation to the amount of hippuric acid (7 to 11 per cent in man; NEUBERG 1924), so that the amount of benzoyl-glycuronic acid is obtained as the difference

between two almost equal figures; the exactness is therefore unsatisfactory.

In the method described below the benzoyl-glycuronic acid is not determined as a difference but instead through direct determination of the benzoic acid bound to benzoyl-glycuronic acid. At first a brief summary of the method is given, and then the necessary details and experiments.

Principle: The benzoyl-glycuronic acid is hydrolyzed in alkaline solution under such conditions that only an inconsiderable amount of hippuric acid is broken down. After saturation with ammonium sulphate and acidification the benzoic acid released is extracted with toluene in a separatory funnel and determined by titration with 0.1-n sodium alcoholate.

Reagents:

10-n sodium hydrate solution.

Concentrated nitric acid.

Ammonium sulphate, dry.

Toluene (the toluene employed can be recovered by being washed with distilled water and distilled and tested as to degree of purity by titration with 0.1-n sodium alcoholate with phenolphthalein as an indicator. One to two drops of alcoholate at most should be used for 200 ml. of toluene).

Thymol blue solution.

Thymol blue paper (3 gm. of thymol blue, 64.5 ml. of 0.1-n NaOH, distilled water to make 1,000 ml.).

0.1-n Na alcoholate made by dissolving about 5 gm. pure metallic sodium in 1 kg. of absolute alcohol; should be kept in a dark bottle fitted with a sodalime tube and a sulphuric acid tube.

It should be titrated against about 100 mg. of benzoic acid pro analysi dissolved in 200 ml. of toluene. Can be kept for at least 3 months.

Phenolphthalein, 1 per cent in absolute alcohol.

Saturated NaCl solution containing 0.5 ml. of concentrated hydrochloric acid per 1,000 ml. of distilled water.

I. *Hydrolysis.* For the determination 100 ml. of urine are poured into an Erlenmayer flask of 300 ml. Ten drops of thymol blue solution are added, after which 10-M natron solution are added in drops under continuous shaking until the colour changes from yellow to green. Then a further 2 ml. of the sodium hydrate solution are added. The mixture is boiled for 2 minutes.

II. *Extraction.*

a. After the sample has cooled to room temperature it is acidified with 3 ml. of concentrated nitric acid (acid against thymol

blue paper). Then 50 gm. of ammonium sulphate are added and dissolved by shaking.

b. To remove carbonic acid which disturbs the determination the sample is transferred to a wash bottle holding about 150 ml. and with a diameter of 4 to 5 cm., after which a rapid stream of nitrogen is led through from the bottom for half an hour.

c. When the carbonic acid has been removed, the sample is transferred to a 500 ml. short stemmed separatory funnel. After that 75 ml. of toluene are added the sample is shaken with strong rotating movements 5 times, every time for 20 seconds. Between each shaking the funnel is left until the toluene has separated. If the shaking is too violent and continuous, a more durable emulsion may be formed. After this first extraction the sample is poured into the original Erlenmeyer flask and the toluene is transferred into a second similar separatory funnel. The first funnel is then rinsed with 10 ml. of toluene, which is afterwards transferred to the second funnel. The extraction is continued by pouring the sample into the first separatory funnel, 75 ml. of toluene are added, and extraction is performed as described above. The sample is finally extracted for the third time with 50 ml. of toluene.

d. When the extraction is finished, all the toluene used in the procedure will thus be found in the second separatory funnel. The urine possibly present is separated from the toluene by repeated rotations of the funnel, and is then drawn off. The toluene is washed twice in the funnel, each time with 100 ml. of the sodium chloride solution; the remaining rinsing fluid is separated off by means of rotatory shaking, and drawn off. The tip of the funnel is washed with distilled water and dried with filtre paper, after which the toluene is transferred to a 500 ml. Erlenmeyer flask. The funnel is rinsed with 10 ml. of toluene which is then added to the rest of the toluene.

III. *Titration.* The benzoic acid is now determined by titration with 0.1-n sodium alcoholate in the toluene. Phenolphthalein dissolved in absolute alcohol (10 drops) is used as an indicator. The titration is continued until a distinctly red colour, remaining for at least one minute, is obtained.

Discussion of Methods.

I. *Hydrolysis*. QUICK (1926) examined the optical activity of benzoyl-glycuronic acid at different pH and was thus able to determine its stability. At a pH lower than 10 to 11 there is no appreciable hydrolysis while, at higher pH, the acid undergoes a rapid break down. In diluted acids benzoyl-glycuronic acid is rather stable at room temperature; when boiled it undergoes hydrolysis in the same way as other coupled glycuronic acids.

Thus, if a rapid and complete hydrolysis of benzoyl-glycuronic acid is desired, this can be performed in alkaline solution. As benzoic acid is volatile with aqueous vapour, there is a risk of losses when boiling it in acid solution. This does not hold for boiling in alkaline solution, where benzoic acid is present as a benzoate. For our purposes the hydrolysis must be performed in such a way that the hydrolyzed amount of hippuric acid is as small as possible. As different urine samples have a varying buffer capacity, the addition of NaOH-solution should always be counted from a fixed point. The reversal of thymol blue from yellow to blue at pH 9—10 can be used as such a point; at this pH the buffering capacity of the urine can be left without consideration.

Experiment No. 1. (Hydrolysis of benzoyl-glycuronic acid after addition of varying amounts of 10-n. sodium hydrate solution. Boiling time 2 minutes.)

For the determination 3,357 mg. of benzoyl-glycuronic acid — corresponding to 1,449 mg. of benzoic acid — and 10,000 mg. of hippuric acid were dissolved in 1,000 ml. urine. Samples of 100 ml. were used for the further determination.

Benzoyl-glycuronic acid in pure form was prepared from dog's urine according to PRYDE and WILLIAMS (1936) (melting-point 170° C).

1. Urine without addition of NaOH-solution (pH 6—7, determined with Lyphan paper)	Benzoid acid found 222 mg/1,000 ml
2. Urine alkalified to reversal with thymol blue (about 0.5 ml. of 10-n solution)	950
3. Urine alkalified to reversal with thymol blue + 0.5 ml. of 10-n solution	1,424
4. Urine alkalified to reversal with thymol blue + 1 ml. of 10-n solution	1,505
5. Urine alkalified to reversal with thymol blue + 2 ml. of 10-n solution	1,586
6. Urine alkalified to reversal with thymol blue + 3 ml. of 10-n solution	1,596
7. Urine alkalified to reversal with thymol blue + 4 ml. of 10-n solution	1,586
8. Urine alkalified to reversal with thymol blue + 8 ml. of solution	1,919

Experiment No. 2. (Hydrolysis of benzoyl-glycuronic acid after addition of 10-n sodium hydrate solution to reversal with thymol blue; after that addition of a further 2 ml. Varying boiling times.)

In 1,000 ml. of urine 1,572 mg. of benzoyl-glycuronic acid (impure preparation), corresponding to 843 mg. of benzoic acid, were dissolved. Samples of 100 ml. were used for the subsequent determination.

	Benzoic acid
1. Sample left at room temperature for one hour	found 666 mg/1,000 ml
2. Just brought to boiling	707
3. Just brought to boiling	681
4. Boiled for half a minute	686
5. Boiled for one minute	686
6. Boiled for one minute	686
7. Boiled for two minutes	695
8. Boiled for 5 minutes	712

Experiment No. 3. (Hydrolysis of hippuric acid after addition of varying amounts of 10-n sodium hydrate solution. Boiling time 2 minutes.)

In 1,000 ml. of urine 10 gm. of hippuric acid were dissolved. Samples of 100 ml. were used for the determination.

	Benzoic acid
1. Urine without addition of NaOH solution (pH 6—7, determined with Lyphan paper) found 2	mg/100 ml
2. Urine alkalified to reversal with thymol blue (about 0.5 ml. of 10-n solution)	6.6
3. Urine alkalified to reversal with thymol blue + 1 ml. of 10-n solution	10.1
4. Urine alkalified to reversal with thymol blue + 2 ml. of 10-n solution	13.1
5. Urine alkalified to reversal with thymol blue + 3 ml. of 10-n solution	16.7
6. Urine alkalified to reversal with thymol blue + 4 ml. of 10-n solution	24.2
7. Urine alkalified to reversal with thymol blue + 6 ml. of 10-n solution	26.3
8. Urine alkalified to reversal with thymol blue + 8 ml. of 10-n solution	50.5

Experiment No. 4. (Hydrolysis of hippuric acid after addition of 10-n sodium hydrate solution to reversal with thymol blue; after that addition of a further 2 ml. Varying boiling times.)

In 1,000 ml. of urine 10 gm. of hippuric acid were dissolved. Samples of 100 ml. were used for the determination.

	Benzoic acid found
1. Sample at room temperature one hour...	3.9 mg/100 ml
2. Brought to boiling	8.1
3. Boiled for half a minute	8.1
4. Boiled for one minute	8.7
5. Boiled for two minutes	10.4
6. Boiled for 4 minutes	14.9
7. Boiled for 6 minutes	16.8
8. Boiled for 8 minutes	19.4

Experiments 1—4 and Figures 1 and 2 show that a complete hydrolysis of benzoyl-glycuronic acid — with a good safety margin — may be obtained by following the method as outlined above and that the simultaneous hydrolysis of hippuric acid is inconsiderable. With respect to the hydrolysis of hippuric acid a correction can be introduced; see below "Specificity of the Method".

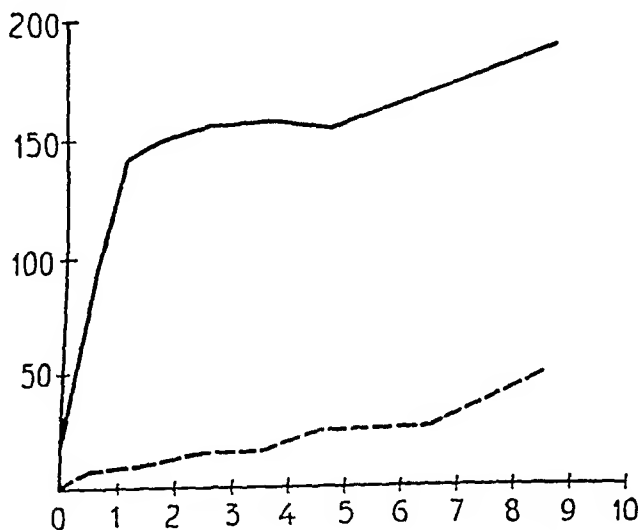


Fig. 1. Hydrolysis of benzoyl-glycuronic acid + hippuric acid ——— and of hippuric acid - - - - - (experiments 1 and 3). Y = mg. of benzoic. X = ml. 10-n NaOH brought to 100 ml. of urine.

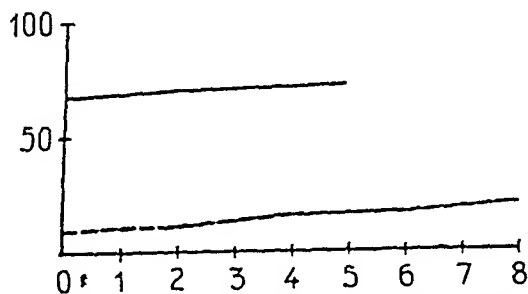


Fig. 2. Hydrolysis of benzoyl-glycuronic acid + hippuric acid ——— and of hippuric acid - - - - - (experiments 2 and 4). Y = mg. of benzoic acid. X = boiling time in minutes. 0 = just brought to boiling.

II. *Extraction.*

b. Carbonic acid passes over into the toluene and gives too high values at the titration with the alcoholate, and must therefore be removed before the extraction. (Experiment No. 5.) The carbonic acid is removed by leading nitrogen through the solution (experiment No. 6).

Experiment No. 5. (Effect of carbonic acid on extraction.)

Solutions containing sodium carbonate, 1-n, 0.1-n and 0.01-n were prepared. Samples of 100 ml. were taken from these solutions, acidified and saturated with ammonium sulphate, and then extracted.

Carbonate content	Consumed alcoholate re-calculated in mg of benzoic acid
1-n.	135
0.1-n.	38
0.01-n.	1

Experiment No. 6. (Removal of carbonic acid with nitrogen.)

For half an hour carbonic dioxide was led through the urine, to which 100 ml. of 10-n sodium hydroxid per 100 ml. had been added in order to obtain a urine with a high content of carbonate. From this urine samples of 100 ml. were taken. These samples were then acidified with 10 ml. of 10-n nitric acid + 3 ml. of concentrated nitric acid, ammonium sulphate was added, and finally nitrogen was led through.

Nitrogen led through for	Consumed alcoholate re-calculated into mg of benzoic acid
30'	12.1
	12.1
60'	13.3
	12.1

Experiment No. 6 shows that even if the sample contains large amounts of carbonate the carbonic acid is removed when nitrogen has been led through for half an hour.

c. As the solubility of benzoic acid in acid urine (reversal of thymol blue to red) amounts to about 0.1 gm/100 ml., and its solubility on toluene to 10.69 gm./100 ml. (at 25° C, SEIDELL 1910) there is a favourable distribution coefficient between toluene and urine.

Experiment No. 7. (Extraction of benzoic acid with toluene.)

Different amounts of benzoic acid (Merck pro analysi) were dissolved in 1,000 ml. distilled water. From this dilution samples of 100 ml. were collected and determined according to the method proposed above.

Added amount of benzoic acid in mg per			
100 ml	501.1	104.7	49.9
Recovered amount of benzoic acid in mg			
per 100 ml	504.9	108.3	53.7
	504.9	107.3	51.5
	504.9	106.2	51.5
	503.8	106.2	50.5
	500.7	106.2	50.5
	499.7	102.0	50.5
	499.7	102.0	50.5
	498.6	99.9	49.1
	496.5	98.9	48.9
Mean values	501.5	104.1	50.8
Standard deviation	± 3.0	± 3.2	± 1.5

Experiment No. 7 shows that total extraction of benzoic acid is achieved through extraction according to the prescriptions in this method.

III. Titration.

As no data concerning the exactness of alcoholate titration or the stability of the alcoholate can be found in the literature, the following experiments have been performed.

Experiment No. 8. (Exactness of titration with 0.1-n sodium alcoholate.)

Varying amounts of benzoic acid (Merck pro analysi) were dissolved in about 200 ml. of toluene. The solution was titrated with phenolphthalein as indicator until a red colour, remaining for at least one minute, was obtained.

mg of benzoic acid added	mg of benzoic acid found	error
153.8	155.1	$\div 1.3$
130.8	131.6	$\div 0.8$
130.4	131.6	$\div 1.2$
125.2	126.9	$\div 1.6$
118.5	119.8	$\div 1.3$
100.9	103.4	$\div 2.5$
93.5	94.0	$\div 0.5$
94.8	96.4	$\div 1.6$
91.7	91.7	$\div 0$
91.5	91.7	$\div 0.2$

Experiment No. 8 shows that the exactness of the alcoholate titration is satisfactory. If the alcoholate is kept in a dark bottle and protected against the water and the carbonic acid of the air, its titer remains practically constant from the day after preparation for at least 3 months.

Specificity of the Method.

If a specific determination of benzoyl-glycuronic acid shall be achieved by the method described above, it is necessary that:

1. No other compounds than benzoic acid pass over into the toluene and give titration values with the alcoholate.

RAIZISS and DUBIN (1915) published a method of determining free benzoic acid in the urine, which was based on a similar extraction with toluene. In normal urines they obtained only titration values lower than 0.1 ml. of 0.1-n alcoholate.

If the carbonic acid has been removed as described above, the titration values found in normal urines before hydrolysis are very low — less than 10 mg. as benzoic acid. These values can probably be ascribed to hippuric acid present in the urine or to benzoic acid formed at unintended hydrolysis of this hippuric acid. Hippuric acid is, however, only slightly soluble in toluene and extraction of pure hippuric acid solutions, therefore yields titration values corresponding to only 2—3 mg. of benzoic acid even if large amounts of hippuric acid have been used.

2. The benzoic acid derives exclusively from benzoyl-glycuronic acid.

The presence of benzoic acid in urine can be explained in two ways: it is either excreted as free benzoic acid, or derived from hydrolysis of hippuric acid and benzoyl-glycuronic in the urine.

According to previous investigations no free benzoic acid appears to be excreted by man. As shown by *i. a.* SEO (1908) hippuric acid may nevertheless under certain conditions be hydrolyzed and give rise to free benzoic acid. SEO found that hippuric acid is split by the action of staphylococci and streptococci, although not by *B. coli*, *typhi*, *paratyphi*, or *pyocyaneus*. Alkaline reaction implies conditions favourable for the growth of bacteria, thus furthering a hydrolysis of hippuric acid in the urine. As proved by SEO (1908) and by J. NEUBERG (1924) this hydrolysis can be prevented by adding a strong acid, chloroform, toluene, or

thymol to the urine. Provided that certain precautions are taken, there is thus little risk of the benzoic acid originating from anything but benzoyl-glycuronic acid. One of the substances enumerated above should be added if the urine is not analyzed immediately.

When hydrolyzing benzoyl-glycuronic acid in an alkaline solution, a certain hydrolysis of hippuric acid, more extensive the greater the amount of hippuric acid in the sample, takes place.

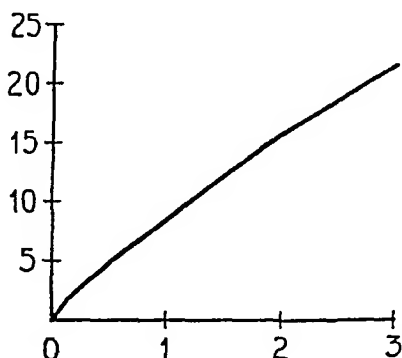


Fig. 3. Correction values for hydrolysis of hippuric acid. Y = mg. of benzoic acid. X = grams of hippuric acid in 100 ml. of urine used for determination.

If the amount of hippuric acid has been determined, *e. g.*, by QUICK's method, the broken-up amount can be calculated. It should then be subtracted from the value determined for benzoyl-glycuronic acid. A correction can be calculated as in Figure 3. This includes values obtained by the passing-over of hippuric acid in the toluene as well as by hydrolysis to free benzoic acid.

If a correction is introduced for hippuric acid and for the benzoyl acid formed by hydrolysis of hippuric acid, our method can be considered practically specific with respect to the determination of benzoyl-glycuronic acid, at least in normal urines.

Exactness of the Method.

Experiment No. 9. For 1,000 ml. of urine an unknown amount of benzoyl-glycuronic acid (impure preparation) and 15 gm. of hippuric acid were dissolved. Determinations of benzoyl-glycuronic acid were made on samples of 100 ml. of this solution. The following values were obtained: 179.9, 176.1, 176.1, 175.4, 172.9, 171.6, 170.9, 170.9, 170.9, and 166.4 mg. calculated as benzoic acid. Mean value: 173.3. Standard deviation: ± 3.9 mg.

Experiment No. 10. Analysis of 100 ml. of a solution containing 15 gm. of hippuric acid per 1,000 ml. gave the following values: 14.9, 14.2, 12.9, 12.9, 12.3, 9.7, 9.7, 9.7, and 9.7 mg. Mean value: 11.8 mg. Standard deviation: ± 2.1 mg.

The value obtained in experiment No. 8 thus includes benzoic acid from benzoyl-glycuronic acid, and small amount of benzoic acid formed through hydrolysis of hippuric acid.

The result of the determination is thus: $173.3 - 11.8 = 161.5$ mg.

Summary.

The paper contains a description of a method of determining benzoyl-glycuronic acid in urine. The method is based on direct determination of the benzoic acid to be found in the benzoyl-glycuronic acid after previous hydrolysis. After the introduction of a correction for hydrolysis of hippuric acid, the method can be considered practically specific as regards the determinations of benzoyl-glycuronic acid in urine.

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From the Clinical Physiological Laboratory at the Caroline
Hospital, Stockholm.

Brain Volume, Diameter of the Blood-Vessels in the Pia Mater, and Intracranial Pressure in Acute Carbon Monoxide Poisoning.

By

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Headache is the earliest and most common symptom of acute carbon monoxide poisoning. This begins with a sensation of pressure in the head, which, a half—two hours after exposure to carbon monoxide or during prolonged exposure, results in a more definite headache accentuated by movements of the body. The first introductive sensation of pressure has long been considered associated with dilatation of the intracranial blood-vessels, apparently first observed in animals by LEWIN (1920), but a fully satisfactory explanation has not been found for the occurrence of the subsequent headache.

FORBES, COBB and FREMONT-SMITH (1924) have attempted to show that this headache is due to an oedema of the brain. They found, on cats exposed to carbon monoxide until severe poisoning occurred, that the brain increased in volume, this was most noticeable shortly after cessation of gassing. They believed that the first introductive increase in volume depended on the dilatation of the blood-vessels of the brain but that the subsequent increase was associated with the occurrence of a brain oedema. They tried in other ways also to support this theory, among others, by observations on man with the help of an ophthalmoscope, but themselves agree that these investigations were not convincing.

Anatomical pathological studies of brains from individuals who have died from severe carbon monoxide poisoning have shown that in these cases oedema of the brain may be present. However, the effects thereby have been the result of extreme carbon monoxide poisoning lasting for several hours or days prior to death. Such an effect is not directly comparable to that of an acute poisoning to the degree where headache is the only or principal symptom.

Since the cause of headache following carbon monoxide poisoning cannot be considered satisfactorily determined, it has seemed worth while to perform experiments on animals in order to re-test the effect of this gas on the brain volume, on the diameter of the blood-vessels in the pia mater and on the state of intracranial pressure.

Method.

Experiments have been made on cats most of them under light dial anaesthesia but a few under short ether anaesthesia which was discontinued shortly after carbon monoxide was administered. During the carbon monoxide poisoning it was not necessary to give ether. A comparison of the observations from these two methods showed no marked differences, therefore light dial anaesthesia was usually used since the animal could be observed during a longer period of time following the administration of carbon monoxide.

A glass window placed in the skull permitted observations of the state of the blood-vessels in the pia mater. Trepanation was accomplished with an electric drill, the bleeding being carefully stopped by pressing plasticine into the diploe tissue. The window, an ordinary watch glass, was fastened to the skull surface with wax, a rather convenient method. In order that the wax should fasten to the bony surface this was first scraped clean of periosteum then washed with hydrogen peroxide and thoroughly dried with alcohol and ether. The edge of the watch glass was waxed and placed against a wax ring around the trepanation opening. Before the wax rings were entirely closed the space between the glass and the brain surface was filled with Ringer's solution. Applied in this way the windows kept for several hours, and, if bleeding occurred under them, the blood could be easily washed away by injecting Ringers' solution under the glass.



Fig. 1. Microphotography of blood-vessels in the pia mater of cat with window mounted in the skull cap. A, before, B, during the effect of carbon monoxide. 1 venal branches, 2 arterial branches. The twisting artery is straightened and expanded also the large vein is somewhat dilated under the effect of carbon monoxide.

Measurements of the diameter of the blood-vessels in the pia mater were made with the help of an ultropak microscope fitted with an ocular micrometer. Changes of the brain volume were obtained by measuring the distance between the surface of the window and a point on the brain surface. The points on each surface were successively observed in the microscope and the displacement of the tube measured on the micrometer scale of the microscope. In this way determinations could be made with an accuracy of $1/20$ of a millimeter.

A water manometer was used to measure the intracranial pressure, it was connected to a cannula fastened to the window. Du-

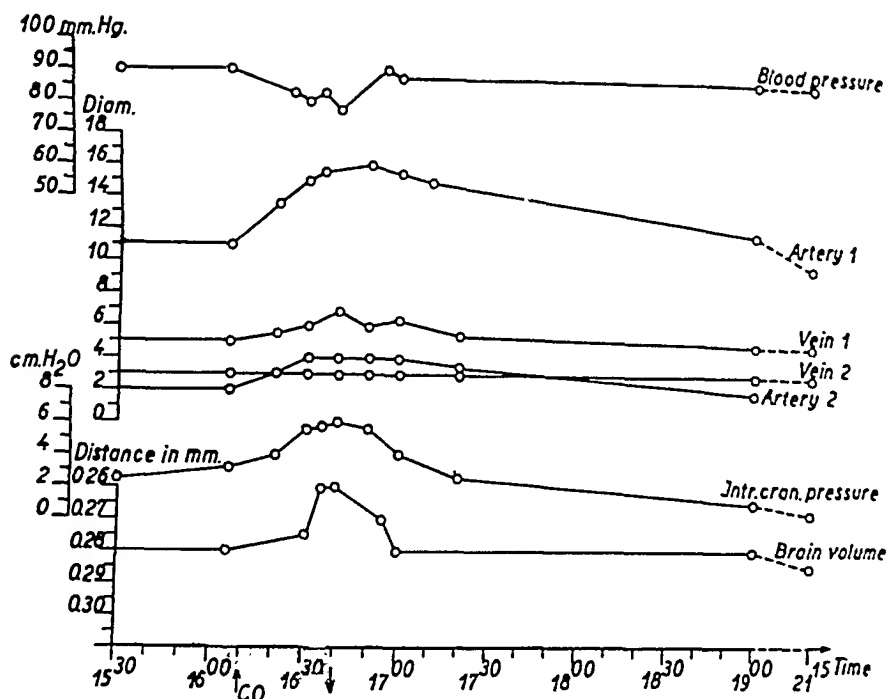


Fig. 2. Effect of carbon monoxide on blood pressure, blood-vessels of the pia mater, brain volume and intracranial pressure. See text for full description.

ring the experiment continuous recordings were made of the blood pressure in the usual manner, and with administration of heparine through the cannula.

The carbon monoxide was produced of formic acid and sulphuric acid.

As a rule the experiments have been so arranged that after the window was mounted in the skull measurements were made and repeated after 30 mins. or more. Then the animal was allowed to breathe air with a carbon monoxide concentration of 0.2—0.3 %, whereupon repeated measurements were made. Carbon monoxide has usually been administered until there is a rapid drop in the blood pressure curve (Fig. 2), which at first showed a continuous fall and a short temporary rise. Continued administration of the gas for another 1—2 minutes causes cessation of breathing and the animal dies if the supply is not immediately stopped and artificial respiration begun. In some experiments CO-exposure has been carried to the point where breathing ceases but in most cases it has been interrupted at the beginning of the critical drop in blood pressure.

Repeated measurements have been made while the carbon

monoxide was administered and 3—5 hours afterwards. When dial was used it has not generally been necessary to give further doses during the observation period. After the animals were rapidly bled to death, the brains have been carefully removed and studied.

Results.

In some preliminary experiments observations were made, through a trepanated opening, of the state of the blood-vessels and the brain volume during and after exposures to carbon monoxide sufficient to produce very marked symptoms of acute poisoning. Dilatation of the blood-vessels was very obvious and the observation of a brain hernia by FORBES, COBB and FREMONT-SMITH (1924) was confirmed. However, it became obvious after the animals were killed and the brains removed that the change of the brain volume was very nearly entirely limited to that part which had been pressed out through the trepanated opening. Since this condition could be explained by the disturbance of the circulation due to the increase of brain volume in connection with the dilatation of the blood-vessels and the subsequent pressure of this part of the brain against the walls of the opening, it was considered necessary to study the effect of carbon monoxide on the diameter of the blood-vessels in the pia mater and on the brain volume through a closed opening in the skull. The following observations were therefore made after a window had been mounted into the skull wall.

Effect of carbon monoxide on the blood-vessels of the pia mater.

In Table 1 results are collected from the measurements of the diameters of the arteries in the pia mater, during and after administration of carbon monoxide. The figures for each animal represent the average for several arterial diameters. In the two columns prior to CO-administration are listed the diameters during two control measurements immediately after and about $\frac{1}{2}$ hour after the mounting of the window; the two columns during CO-administration give the values 5—10 mins. after initial exposure and at the maximal effect. As appears from Table 1 the average arterial diameter markedly increases under the effect of carbon monoxide and with 40—80 % (see also Fig. 1). The dia-

Table 1.
Arterial diameter during CO-poisoning.

Animal number	Date	No. of observations	Diameter in 1/10 mm.					
			Before CO		During CO		After CO	
			1	2	1	2	30-75 min.	2 $\frac{3}{4}$ -4 $\frac{1}{2}$ hrs.
1.....	6. 8.	3	10.5	9.4		15.5		8.6
2.....	29. 8.	8	8.2	7.5	9.3	12.5	8.7	8.0
3.....	1. 9.	4	11.5	10.8		18.0	12.5	10.5
4.....	8. 9.	4	9.2	9.8		13.9	9.2	8.5
5.....	28. 10.	3	11.8	12.0	14.6	19.0	16.8	11.4
6.....	7. 11.	4	6.9	6.8	7.9	11.6	7.8	
7.....	14. 1.	4	9.6	9.5	11.6	13.5	9.2	7.9

Table 2.
Venal diameter during CO-poisoning.

Animal number	Date	No. of measurements	Diameter in 1/10 mm.					
			Before CO		During CO		After CO	
			1	2	1	2	30-75 min.	2 $\frac{3}{4}$ -4 $\frac{1}{2}$ hrs.
1.....	6. 8.	6	18.5	18.5	20.2	20.5		15.8
2.....	29. 8.	5	9.7	12.8	13.2	13.4	13.7	11.8
3.....	1. 9.	4	7.5	7.8		8.8	8.5	7.8
4.....	8. 9.	5	10.6	10.2	11.0	10.7	10.2	8.6
5.....	28. 10.	3	8.4	8.0	8.4	9.0	9.4	8.4
6.....	7. 11.	3	15.4	16.6	16.5	16.6	17.2	
7.....	14. 1.	4	14.0	14.2	15.4	15.8	14.8	12.8

meters of the thicker arteries increased percentually less (25-50 %) than the thinner ones, which sometimes were doubled.

This dilatation of the arteries continues in most of the experiments for 30-75 minutes following cessation of CO-administration, sometimes even longer, but 2 $\frac{3}{4}$ -4 $\frac{1}{2}$ hours after exposure it seems to have reverted to a slight decrease in diameter.

The diameters of the veins, on the other hand, are only slightly changed, but as a rule both the thicker as well as the thinner ones are somewhat dilated. This dilatation slowly reverts and after 2 $\frac{3}{4}$ -4 $\frac{1}{2}$ hours following CO-administration the diameter is often less than before.

Even the thinnest blood-vessels on the brain surface are dilated as indicated by the redder tone of this surface. This change in color which is often marked would seem to occur not only as the result of a dilatation of previously opened blood-vessels but also by an opening of previously closed vessels. In several cases it could be shown that anastomoses have been opened between two arteries.

The effect of carbon monoxide on the brain volume.

It has been possible from determinations of the distance between a point on the surface of the window and one on the brain surface to follow the changes in the brain volume without altering the pressure within the skull cavity.

In Table 3 the values obtained by these determinations have been collected and it appears that this distance decreases with 8—55 % as long as the carbon monoxide is administered and increases slowly until after $2\frac{3}{4}$ — $4\frac{1}{2}$ hours when it generally is the same as before.

Table 3.

Distance between window surface and brain surface in mm.

Animal number	Date	Before CO		During CO		After CO	
		1	2	1	2	30—75 min.	$2\frac{3}{4}$ — $4\frac{1}{2}$ hrs.
1.....	6. 8.	2.9	2.9	2.7	2.7	2.9	2.9
2.....	29. 8.	1.8	1.9	1.7	1.6	1.5	1.7
3.....	1. 9.	2.9	2.8		2.4	2.4	2.9
4.....	8. 9.	2.1	2.0	1.9	1.5	1.7	2.0
5.....	28. 10.	2.8	2.8	2.7	2.6	2.8	2.85
6.....	7. 11.	2.0	2.0	1.9	1.7	2.0	
7.....	14. 1.	2.75	2.7	2.5	2.5	2.6	2.7

At the end of the experiment the animals were bled to death and the brains removed. In only one case it was possible to demonstrate a swelling of the brain (Experiment 29. 8).

The effect of carbon monoxide on the intracranial pressure.

In two experiments the intracranial pressure was measured during the entire period of observation and the values thus obtained are collected in Table 4. These values must be under-

Table 4.
Intracranial pressure in cm H₂O.

Animal number	Date	Before CO		During CO		After CO	
		1	2	1	2	30—75 min.	2½—4½ hrs.
1.....	28. 10.	4.75	5.0	6.0	7.2	4.4	2.3
2.....	7. 11.	5.8	6.9	8.8	11.0	0.8	

stood as correct in relation to each other but not absolutely, since no particular attempt was made to obtain an exact adjustment of the manometer level in relation to the cannula point under the window. This was not considered important in these experiments. As seen in the Table the intracranial pressure greatly increases during the exposure to CO but decreases rapidly upon the cessation of CO-administration. Already 30—75 minutes after CO-administration has ceased the pressure is lower than before and continues to decrease.

The time relations between changes of the diameter of the blood-vessels in the pia mater, the brain volume and the intracranial pressure.

The time relations between changes in the diameter of the blood-vessels in the pia mater, the brain volume and the intracranial pressure have to a certain extent already become apparent. Dilatation of the blood-vessels and increase in the brain volume accompany each other and only slowly decrease. The diameter of the blood-vessels and the brain volume return to their initial state first a few hours after the administration of carbon monoxide. The intracranial pressure rises with the increase in diameter of the blood-vessels and in brain volume but falls much more rapidly than the comparable reversion of the latter. This is also apparent from a closer analysis of the separate experiments such as are illustrated in Fig. 2. The rapidity with which these changes occur following the instigation of CO-administration may also be seen on the diagram thus indicating the sensitivity to carbon monoxide of the mechanism regulating the blood supply to the brain. The marked drop in intracranial pressure following CO-exposure is not explained by changes in

the brain volume and neither, as shown in Fig. 2, by a decrease in blood pressure, but seems to be dependent on conditions not apparent from the observations which have been made here.

Discussion.

The experiments have shown that the brain oedema following an acute carbon monoxide poisoning, which can be demonstrated by observations through a trepanated opening does not generally have its counterpart if the skull cavity is closed. Observations through the mounted window showed that the increase of the brain volume is accompanied by the dilatation of principally the arteries and the thinner blood-vessels in the pia mater and is probably also dependent on the dilatation of the blood-vessels in the inner brain, which was observed earlier with respect to the thinner blood-vessels (SJÖSTRAND, 1935). This swelling of the brain slowly recedes, however, at the same time as the diameter of the arteries in the pia mater become reduced. Only in one experiment with closed opening in the skull, could also the presence of a brain oedema be demonstrated after the animal was killed.

The explanation of the residual brain oedema observed through a trepanated opening may be that the brain swelling accompanying dilatation of the blood-vessels is greater when the intracranial pressure is zero, which results in a "pinching" of that part of the brain substance in the trepanated opening. This causes a disturbance of the circulation in that part of the brain with subsequent appearance of a local oedema. The observations made by FORBES, COBB and FREMONT-SMITH (1924) after trepanation must not, consequently, be transferred to the conditions which exist with an unopened skull.

Even if it is well known in pathology that a fatal carbon monoxide poisoning can be the cause of a brain oedema in man, it is not thereby proved, that this condition is the fact in cases of slight or moderate poisoning and that it is the reason for the headache present in the latter case. It is therefore justifiable to assume that for the present no acceptable explanation has been given for the headache usually occurring after an acute carbon monoxide exposure.

From the results found in this investigation the following ex-

planations may be given for the headache symptom of carbon monoxide poisoning. During the absorption of carbon monoxide by the blood and already at low concentrations of carboxyhaemoglobin the blood-vessels of the skull cavity, particularly the arteries and capillaries, within as well as outside the brain, are dilated. This results in a swelling of the brain and a pressure rise in the skull cavity producing a sensation of "pressure in the head". When the stroke volume of the heart and thereby the pulse amplitude is increased by exercise, the distension of the arterial walls produces a sensation of pain. This is perhaps particularly true of the a. meningea media which is well supplied with pain end-organs, thus explaining the fact that the accentuated headache is usually located in the temporal region. After exposure the intracranial pressure drops and the sensation of pressure also disappears. The remaining dilatation of the arteries, however, may still cause the factors influencing the blood supply to the brain, such as, exercise, change in position of the head, to give rise to a sensation of throbbing pain. In connection with the subsequent drop in intracranial pressure below the initial level, it is possible that tension is exerted on the walls of the vein sinuses which may produce a sensation of pain. This would explain why the subsequent headache has a different character from the original feeling of pressure and that it is located to the top of the head, forehead or neck varying for different individuals.

Finally it may be pointed out that the pressure rise in the skull cavity due to absorption of carbon monoxide causes a corresponding increase in the capillary pressure in the brain. This is inclined to counteract the improvement in the blood flow which a dilatation of the vessels would seem to imply. Naturally if the intracranial pressure becomes very high the blood flow may instead be retarded which would then contribute towards an impairment of the oxygen supply to the brain tissues. Within those parts of the brain where adaptation of the blood-vessels for some reason is not possible, *e. g.* by arteriosclerotic changes, traumatic injuries, the rise in intracranial pressure causes an impairment of the conditions for the blood flow which is not compensated for by dilatation of the blood-vessels. Therefore an impairment of the oxygen supply at relatively low concentrations of carboxyhaemoglobin may occur within these areas of the brain. This may be an explanation for the very low tolerance to carbon

monoxide which some individuals display with respect to certain functions of the central nervous system (SJÖSTRAND, 1942).

Summary.

Using a simplified technique, a glass window was mounted in the skull wall of cats under light dial or ether anaesthesia, thereafter the effect of carbon monoxide administered via the lungs was observed on the diameter of the blood-vessels in the pia mater, on the brain volume and on the intracranial pressure. The diameter of the blood-vessels was measured with an ocular micrometer, the brain volume by determining the distance between the brain surface and the window surface, the intracranial pressure by a water manometer connected to a cannula fastened to the window.

During absorption of carbon monoxide the diameter of the blood-vessels in the pia mater increases, particularly the arteries and the capillaries, the brain swells and the intracranial pressure rises. After the administration of carbon monoxide the brain volume and the diameter of the blood-vessels slowly decrease approximately to their original value. On the other hand the intracranial pressure reverts more rapidly and falls considerably below the initial level. These observations are illustrated in Fig. 2.

The above observations seem to explain the presence of the headache accompanying carbon monoxide poisoning and the very low tolerance to this gas which some individuals display with regard to certain functions of the central nervous system.

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From the Department of Physiology, University of Lund.

Action of n, n-Dibenzyl-Chloroethylamine (Dibenamine) on the Effect of Sympathetic Secretory Impulses to the Submaxillary Gland of the Cat.

By

BÖRJE UVNÄS.

Received 5 March 1948.

Last year NICKERSON and GOODMAN introduced a new sympatholytic drug, dibenamine. According to these authors the drug reduces or annuls sympathetic excitatory effects on smooth muscle. Dibenamine is assumed to block excitatory effects of the transmitter at sympathetic nerve endings but not to interfere with the release of the transmitter. As it seemed of interest to investigate the action of the new drug on the effect of sympathetic secretory impulses, the following experiments were made.

The experiments were performed on cats under chloralose anaesthesia. Sherrington electrodes were placed on the sympathetic trunk in the neck and on the lingual nerve just proximal to the point where the chorda tympani nerve branches off. Both nerves were ligated proximal to the electrodes. Electrical stimuli were delivered by a thyratron stimulator. The nerves were stimulated for 30 seconds and the secretion, measured in drops from the cannulated submaxillary duct, was registered by a drop recorder. Between every stimulation the gland was allowed to rest for at least 5 minutes. The blood pressure was recorded in the femoral artery by a mercury manometer. Dibenamine was given intramuscularly, intraperitoneally or slowly intravenously.

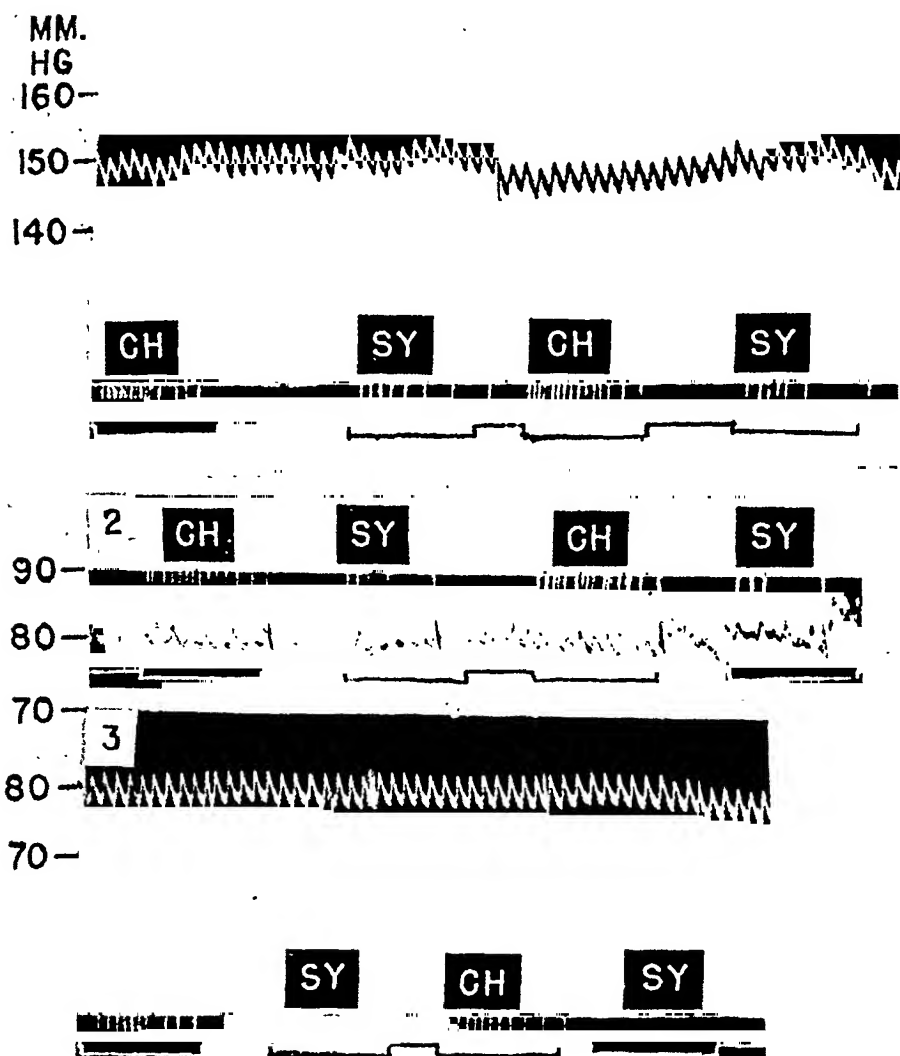


Fig. Cat 3 kg. Chloralose. Salivary secretion during stimulation of the chorda tympani nerve (Ch) and the sympathetic trunc (Sy).

From above: Blood pressure, secretion, stimulation.

1. Before dibenamine.

2. 30 minutes after dibenamine 10 mg/kg intravenously.

2. 30 " " a repeated dose of dibenamine 10 mg/kg intravenously.

Results.

In experiments performed on eight cats the secretory effect of the stimulation of the sympathetic trunk was completely abolished by dibenamine 10—20 mg per kg body weight. This blocking effect did not appear instantly but increased gradually and was

maximal in about an hour. The pupillary dilatation and the contraction of the nictitating membrane elicited by the stimulation of the sympathetic nerves considerably or completely disappeared in the same time. The secretory response to chordal stimulation was not abolished but was usually reduced by 20—30 per cent. It is not clear whether this reduction indicates a specific effect of dibenamine. It might be secondary to the considerable blood pressure fall caused by dibenamine on chloralozed cats. The blood pressure usually fell to and remained at 80—100 mm of mercury. The fig. shows one of the experiments.

Summary.

Dibenamine in doses of 10—20 mg per kg body weight completely abolishes the salivary secretion elicited by sympathetic stimulation.

Reference.

NICKERSON, M. and L. S. GOODMAN, J. Pharmacol. 1947. 89. 167.

From the Department of Physiology, University of Lund.

The Chemical Transmission of Vasoconstrictor Impulses to the Hind Limbs and the Splanchnic Region of the Cat.

By

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Received 5 March 1948.

The chemical nature of the transmitter at adrenergic nerve endings has for many years been a matter of dispute. As adrenaline in its biological action imitates many sympathetic effects this substance was early suspected to play a part in the mediation of the effect of sympathetic impulses (ELLIOT 1904).

The experimental evidence proving the existence of a humoral transmission mechanism at sympathetic nerve endings was given by LOEWI (1921). In a later paper Loewi (1936) concluded from experiments on the frog heart that no experimental evidence contradicted the idea that "Sympathicusstoff" is identical with adrenaline. CANNON and URIDIL as early as 1914 made the important discovery that on stimulation of the hepatic nerves in cats, a substance appeared in the blood stream inducing acceleration of the denervated heart and a rise of blood pressure. There were no reports on the nature of this blood borne principle until 1931 when in the first of a series of papers CANNON and his group showed that stimulation of sympathetic nerves, in addition to its local action, elicited remote excitatory effects on denervated organs such as the heart, smooth muscle and salivary gland. The term sympathin was used to designate the effector substance. CANNON and BACQ (1931) suggested that sympathin and adrenaline possibly were identical.

CANNON and ROSENBLUETH (1933, 1935), however, observed differences in the biological effects of sympathin and adrenaline. An attempt to overcome the difficulties involved in the hypothesis that sympathetic effects were mediated by one transmitter, was made by CANNON and ROSENBLUETH (1933) with the postulate of a sympathin E and I. According to their conception, sympathetic impulses release a mediator substance — possibly adrenaline — that in the effector cell is further elaborated forming new active principles. "Two kinds of sympathin are produced, sympathin E, excitatory, produced by structures stimulated, and sympathin I, inhibitory, produced by structures inhibited by sympathetic impulses." The remote actions observed were, according to their theory, produced not by the mediator substance itself but by principles formed in the effector cells. CANNON and LISSÁK (1939) and LISSÁK (1939) in extracts of adrenergic nerves and various organs found an agent with biological and chemical properties similar to those of adrenaline. These findings were considered "to be consistent with their idea that adrenaline is liberated at the endings of adrenergic fibres, and that sympathin, which escapes from the stimulated region into the blood stream, is the adrenaline which has been modified by the affected cells".

CANNON and ROSENBLUETH's theory has not been generally accepted. BARGER and DALE (1910) investigated the biological properties of several sympathomimetic amines. They found that arterenol (nor-adrenaline) imitated sympathetic effects more closely than did adrenaline. BACQ (1934) suggested that adrenaline might be identical with sympathin I and nor-adrenaline with sympathin E. Recently EULER (1945, 1946) in a series of papers has reported that extracts from adrenergic nerve fibres and from tissues innervated by such fibres contain a specific sympathomimetic principle. This principle shows biological and chemical properties more similar to nor-adrenaline than to adrenaline. From his studies EULER concludes that nor-adrenaline might be the adrenergic transmitter. On the other hand GADDUM and KWIATKOWSKI (1938, 1939) and GADDUM, JANG and KWIATKOWSKI (1939) conclude from studies on the chemical and biological properties of the substance liberated by adrenergic nerves in the rabbit's ear that their experiments "leave no doubt that the active substance is adrenaline". Recently GERNANDT and ZOTTERMAN (1946), investigating the influence of ergotamine

on the vasopressor response to adrenaline and stimulation of the splanchnic nerves in the cat, conclude that adrenaline might be the adrenergic transmitter.

Ergotamine has been used by many investigators to study problems concerning the mechanism of vasomotor control. DALE (1913), CANNON and ROSENBLUETH (1935), BÜLBRING and BURN (1935) used this drug to show the existence of sympathetic vasodilators in the dog and cat. However, the validity of the conclusions drawn from observations on ergotaminized animals has been questioned. For instance, it has been discussed whether the modification caused by ergotamine on vasoconstrictor effects produced by stimulation of sympathetic nerves is due to a blocking of excitatory responses and a consequent unmasking of a vasodilator action or whether the results obtained represent a reversal of the response of effector cells to the sympathetic mediator, as is the case with injected adrenaline.

NICKERSON and GOODMAN (1947) introduced a new sympatholytic drug, N,N-dibenzyl-chloroethylamine (dibenamine). According to their claims the drug reverses the vasopressor responses elicited by adrenaline, by stimulation of the splanchnic nerves, anoxia, and the nicotine action of carbaminoylcholine on atropinized cats. In comparison to ergotamine, dibenamine was considered to have "the advantages of specificity, potency and prolonged action". As a substance with these properties seemed to us to offer new possibilities for the study of some problems concerning the transmitter mechanism at vasomotor nerve endings, a series of experiments were started, the first parts of which are presented in this paper.

PART I.

Action of Dibenamine on Vasomotor Responses Elicited by Adrenaline, and by Electrical Stimulation of the Lower Abdominal Sympathetic Chain.

Experimental.

The experiments were performed on eviscerated cats under chloralose (60 mg/kg) or combined chloralose-urethane anaesthesia (50 mg chloralose and 500 mg urethane per kg body weight). The liver with its arterial supply, the kidneys and the adrenals were left intact. The animal was partly divided into an upper and a lower half by

mass ligation of the muscles and the skin at the height of the fifth lumbar vertebra. The sympathetic chains were cut at the height of the second lumbar vertebra and carefully dissected free down to the fifth lumbar vertebra. The white and grey communicant fibres of this part of the chains were severed. The abdominal aorta and the lower caval vein were freed and all branches from about two centimeters above the dividing plane down to the bifurcation of the large abdominal vessels were ligated. In this way the cat was divided into two parts, the only connections between the upper and the lower part being the vertebral columna with the spinal medulla, the vertebral vessels, the aorta and the lower caval vein.

The blood flow through the hind limbs was measured by a Gaddum recorder (GADDUM 1929), the outflow from the hind limbs coming from the distal portion of the ligated caval vein and the inflow from the Gaddum recorder entering through the proximal end of this vein. Intraarterial injections were made into the stump of the severed inferior mesenteric artery. To obtain reliable injection conditions all injections were made with a constant volume (0.2 ml) at 37 °C. As the substances injected were dissolved in physiological saline, frequent control injections of saline were given. The blood pressure was recorded in the brachial artery by a mercury manometer. The sympathetic trunks were stimulated by a Collison electrode (COLLISON 1934), usually by stimuli from a thyatron stimulator, capable of delivering impulses of varying frequency and voltage. When not otherwise stated the frequency of stimuli was 60/sec. Dibenamine was given slowly intravenously by a perfusion apparatus. Heparin was used as an anticoagulant.

Results.

Intraarterial injection of 0.10—10 γ adrenaline usually caused a pronounced vasoconstriction. A decrease in the blood flow through the hind limbs and a rise of blood pressure occurred. A rather frequent observation was the wellknown vasodepressor effect of small adrenaline doses (less than 1 γ); an initial increase of the blood flow occurred, followed by a more or less marked decrease.

Sympathetic stimulation almost regularly elicited a marked vasoconstriction. As will be discussed in a later report vasodilatation could be obtained with stimuli of certain frequency and strength.

Dibenamine caused a fall of blood pressure, that usually was lowered to and remained at about 100 mm Hg. The vasopressor response to intraarterial injection of adrenaline was reversed a few minutes after some mg dibenamine. The vasopressor response to sympathetic stimulation resisted dibenamine considerably

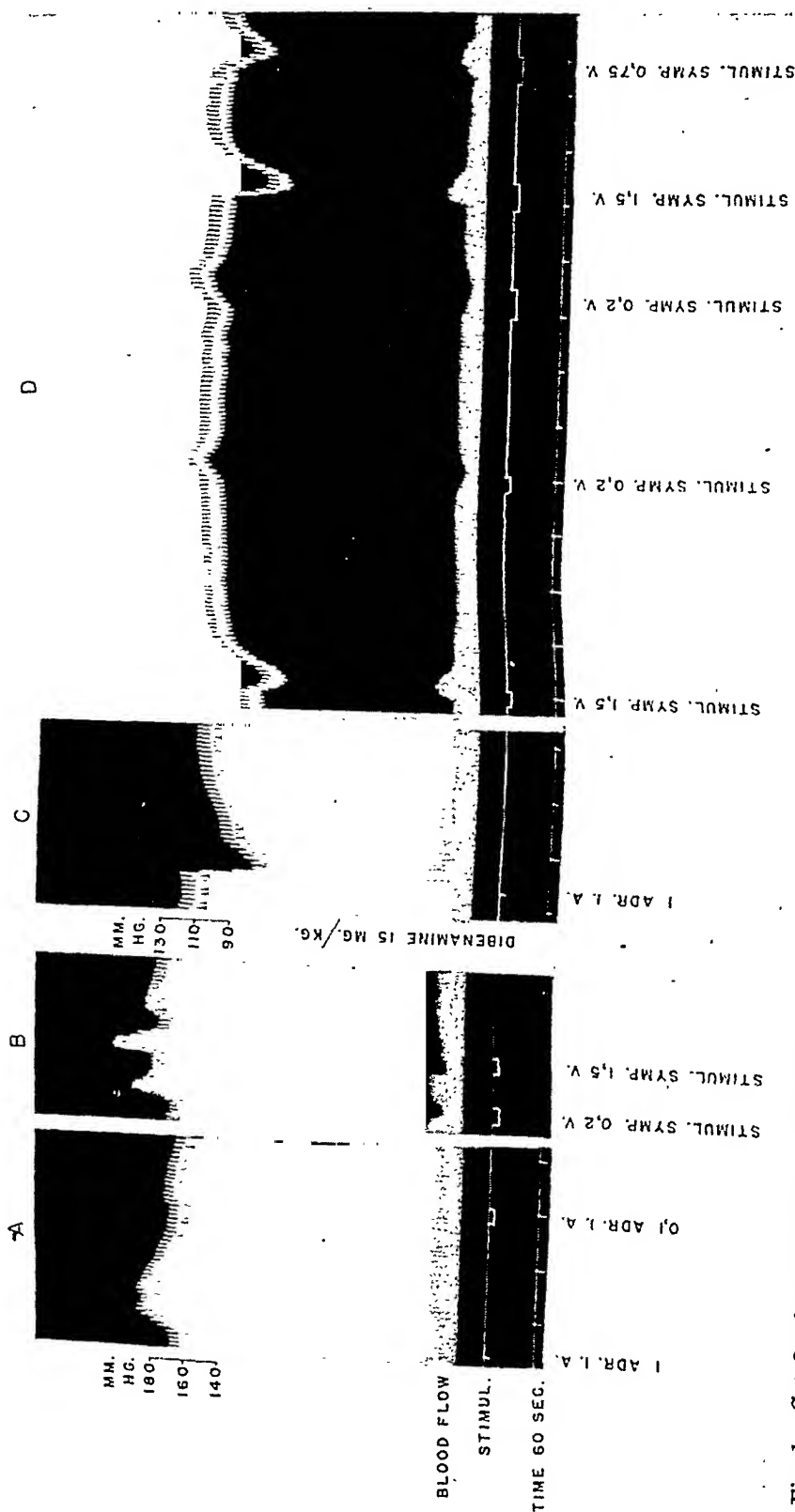


Fig. 1. Cat 3.2 kg. Chloralose. Vasomotor responses to adrenaline, and to stimulation of the lower abdominal sympathetic chain by stimuli of varying intensities.

From above: Blood pressure, blood flow through the hind limbs, stimulation and injection marks, time 60 seconds.

A) responses to adrenaline before dibenamine. B) responses to symp. stim. before dibenamine. C) response to adrenaline after dibenamine (15 mg/kg). D) responses to symp. stim. after dibenamine.

Note the persisting vasoconstrictor responses to weak stimuli.

longer but after 10—20 mg per kg body weight it changed to a vasodepressor response. The reversal was maximal in 30—60 minutes and persisted as long as the experiment lasted, that is for at least 6 hours.

However, a rather puzzling vasomotor response to stimuli of certain frequency and strength was observed on some cats treated with dibenamine. An observation of this kind is shown in fig. 1. Intraarterial injections of 0.1 and 1 γ adrenaline elicited a vasoconstriction. The blood flow through the limbs diminished concomitantly with a rise of the general blood pressure. The sympathetic chains were stimulated by square formed stimuli of ten milliseconds duration and a frequency of 40 per sec. The nerves were stimulated first with a low (0.2 V), and then with a higher intensity (1.5 V). Marked vasoconstrictor effects were obtained. Dibenamine 15 mg/kg was then given. The previous vasopressor effect of adrenaline was completely reversed. The blood flow in the hind limbs increased concomitantly with a fall of the general blood pressure. Repeated stimulation of the nerves showed that stimuli of the higher intensity now elicited a pronounced vasodilatation. The weaker stimuli still caused a vasoconstriction. These differences between the effects of stimuli of higher and lower intensity persisted for more than an hour, a time long enough for the sympatholytic effect of dibenamine to become maximal.

Comments.

NICKERSON and GOODMAN observed on cats that dibenamine reverses the vasoconstriction elicited by stimulation of the splanchnic nerves, by anoxia and by the nicotine action of carbaminoylcholine. They interpret these observations on the basis of the sympathin theory. Dibenamine is considered to act on the effector cells preventing the excitatory responses to sympathin E. The vasodepressor effects observed under the influence of dibenamine are believed to be due to the unmasked action of sympathin I. In our view NICKERSON and GOODMAN's observations do not justify decisive conclusions as to the nature of the transmitter mechanisms at vasomotor nerve endings. Using their experimental procedures it is impossible to decide to what extent the vasodilator effects observed are due to the activation of vasodilator nerve fibres or to the peripheral action of metabolites accumulated during anoxia.

Our experiments confirm NICKERSON and GOODMAN's observation that dibenamine reverses the vasoconstrictor action of adrenaline and they partly confirm their observation that the drug reverses vasoconstrictor responses to stimulation of sympathetic nerves. The fact that in our experiments, under the influence of dibenamine weak stimuli still elicit vasoconstriction while stronger stimuli elicit vasodilatation indicates that the vasomotor responses are probably due to the concomitant stimulation of two different types of nerves. Weak stimuli predominantly elicit vasoconstrictor impulses, the action of which is not completely blocked by dibenamine. Stronger stimuli in addition activate vasodilator fibres, the vasodilator effect being unmasked by the partial blocking of the constrictor response. The fact that the constrictor response to weak stimuli is simply reduced but not reversed to a vasodilator response, in our view does not favour but rather contradicts the idea that adrenaline is the transmitter at vasoconstrictor nerve endings.

A further analysis, by the use of sympatholytic drugs, of the biological properties of the transmitter substance at vasoconstrictor nerve endings, requires experimental procedures where exclusively vasoconstrictor nerves are activated. Our experiments were therefore extended to an investigation on the effect of dibenamine on *reflex* vasoconstrictor responses obtained by activation of vasoconstrictor centres, for instance by lowering the blood pressure. As a vasopressor response elicited in this way involves the activation of nervous pathways with synapses in the central nervous system and peripheral ganglia, we found it necessary to record the action of dibenamine on the involved nervous pathways, especially in view of reports that another sympatholytic drug, ergotamine, interferes with the action of baroreceptive impulses from the carotid sinus. According to EULER and SCHMITERLÖW (1944) this action of ergotamine is localized in the CNS.

PART II.

Site of Action of Dibenamine.

A. Action on Sympathetic Ganglia.

Technique.

We experimented on the superior cervical ganglion, the contraction of the nictitating membrane serving as an indicator for

sympathetic postganglionic discharge. The ganglion was prepared according to KIBJAKOW (1933). The jugular vein and the common carotid artery with all branches except those supplying the ganglion were ligated.

To obtain a concentration of dibenamine in the perfusate comparable to that obtained in the blood when administered to the whole animal the ganglion was perfused from a donor cat to which the drug was given. Cross-circulation was established in the following way. Arterial blood from one of the common carotids of the heparinized donor cat was directed into the proximally ligated common carotid artery of the recipient cat. The outflow from the ganglion was collected through a cannula in the jugular vein, the perfusion thus being under permanent control. With this technique dibenamine did not reach the nictitating membrane of the recipient. Stimulation of the sympathetic trunk in the neck was performed by a Collison electrode. Electrical stimuli were obtained from a thyatron stimulator.

Results.

Responses of the nictitating membrane to praeganglionic stimulation still remained unaffected two hours after the administration of dibenamine 20 mg/kg to the donor. Acetylcholine was given intraarterially to the ganglion. The injections evoked strong contractions of the nictitating membrane at the beginning as well as at the end of the experiment proving that dibenamine did not interfere with the action of acetylcholine and that the ganglion remained in good condition. Three experiments all gave uniform results.

B. Action of Dibenamine on Transmission in CNS.

The effect of dibenamine on the transmission in the central nervous pathways concerned with eliciting reflex vasoconstriction in the hind limbs was studied in cross-circulation experiments. These were arranged in such a way that dibenamine did not reach the endings of the activated vasoconstrictor nerves.

Mode of preparation.

Recipient.

The recipient cat was eviscerated as described on page 367. The animal was then divided into an upper and a lower part at the height of L 5 by careful ligation of the abdominal wall and all the longitudinal muscles of the back until the vertebral columna was reached. By this procedure all vessels connecting the two parts of the animal except the aorta, the lower caval vein and the vessels in the vertebral columna were obstructed.

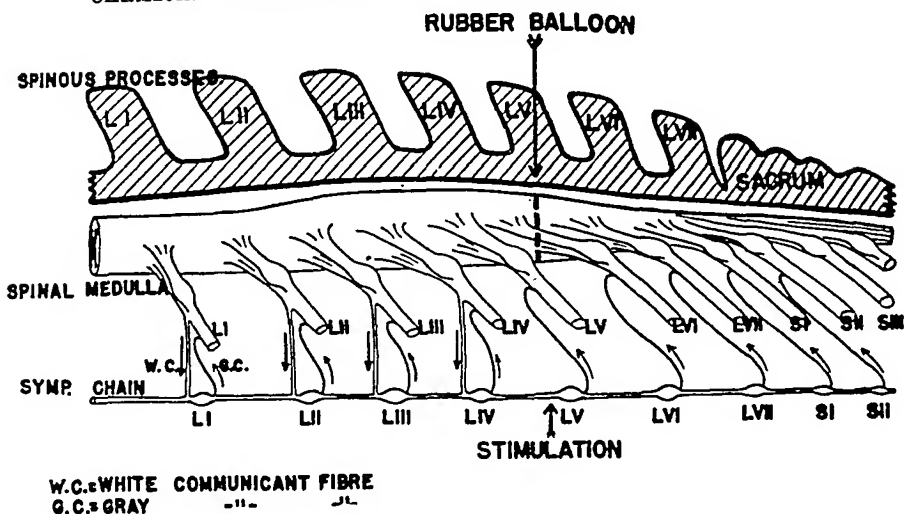


Fig. 2. Schematic drawing illustrating the sympathetic innervation to the hind limbs of the cat and the point where the rubber balloon was inserted and inflated in order to obstruct spinal vascular and nervous connections between the fore-quarters and the hind limbs of the recipient.

Injury to the abdominal sympathetic chains was carefully avoided. The nerves were protected from drying and cooling by covering them with cotton wool soaked in blood and warming them with a heat lamp. A hole was drilled in the dorsal part of the fifth lumbar vertebral arch. A cannula with a small rubber balloon tied round its tip was inserted through this hole into the spinal canal. By inflating the balloon with water under high pressure, the spinal medulla, and intraspinal vascular connections between the two parts of the animal were blocked. As the preganglionic sympathetics innervating the hind limbs emanate from the first four lumbar segments and the dividing plane is situated at the lower end of the fifth lumbar vertebra, the sympathetic outflow to the hind limbs remains intact (fig. 2). *The abdominal sympathetic chains thus form the only nervous connection between the two parts of the animal.*

Donor.

The donor animal was eviscerated as described above. A few centimeters of the aorta, and the lower caval vein were dissected free for the insertion of cannulas.

Establishing cross circulation (fig. 3).

The cats were heparinized with 0.1 mg heparin per kg intravenously. The aorta and the lower caval vein of both animals were ligated at the height of L 5. The proximal end of the aorta of the donor was connected with the distal part of the aorta of the recipient. The outflow from the hind limbs of this animal was

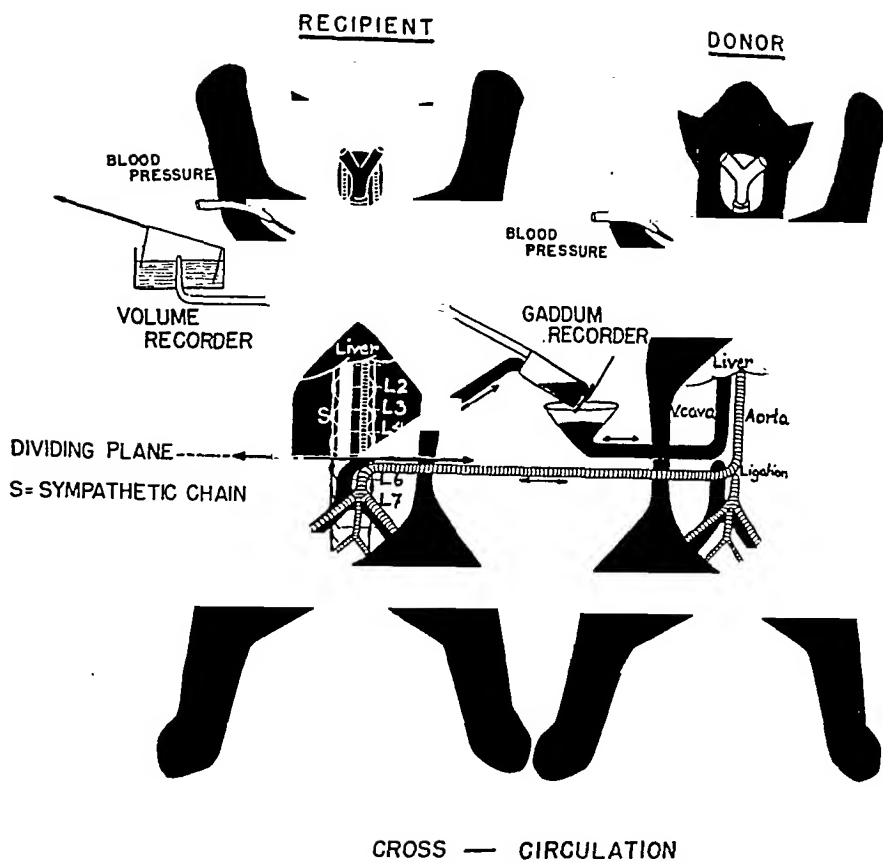


Fig. 3. Schematic drawing illustrating the cross-circulation arrangement.

directed to a Gaddum recorder through a cannula in the lower caval vein distally to the ligature.

From the recorder the venous blood was drained to the proximal part of the ligated caval vein of the donor. *By this arrangement the isolated hind part of the recipient received blood solely from the donor whilst its vasomotor control was supplied via the sympathetic chains of the recipient, these being the only nervous pathways for impulses from the CNS.* The absolute vascular separation of the two parts of the recipient was evident from the fact that no indication of dibenamine action could be observed in the undibenaminized part of the animal even in experiments lasting 5—6 hours.

The blood pressures of both animals were recorded by mercury manometers connected to the brachial arteries. Intravenous injections were made into the brachial veins. Intraarterial injections to the hind limbs of the recipient were given into the cannulated stump of the inferior mesenteric artery.

Vasoconstrictor discharges to the hind limbs were elicited by lowering the blood pressure in the carotid sinus region by clamping

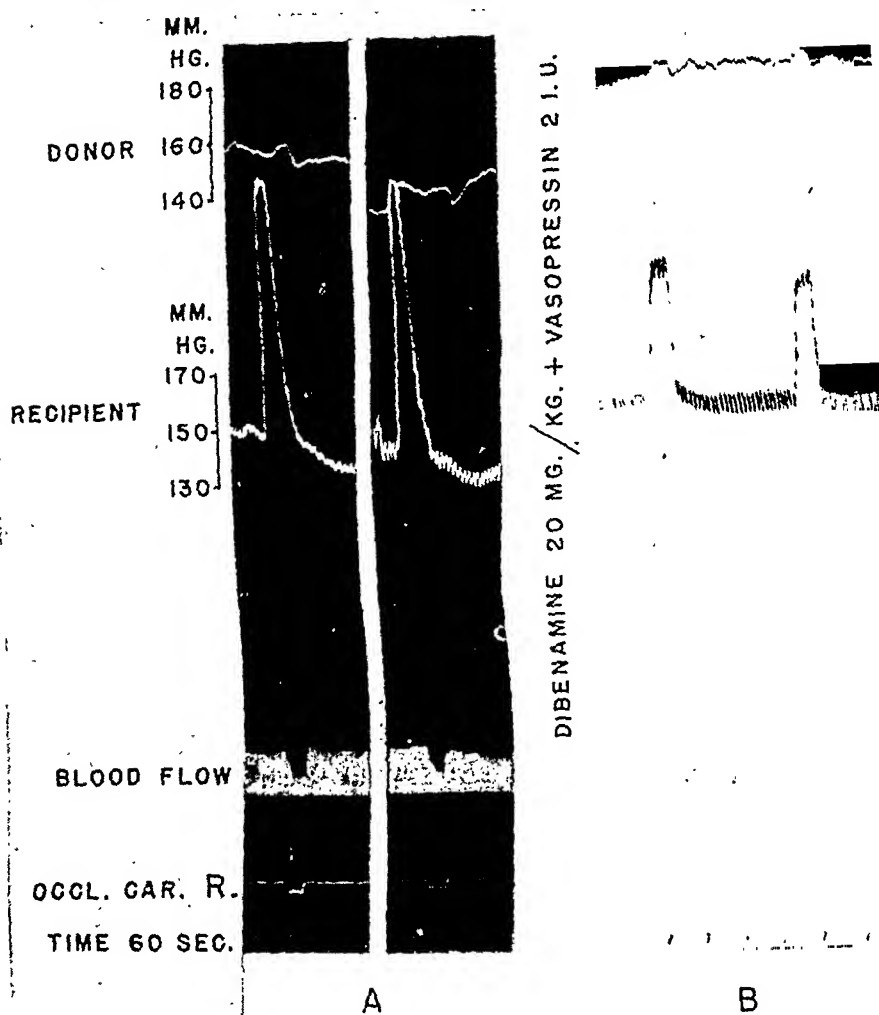


Fig. 4. Cross-circulation. Donor 3.8 kg. Recipient 3 kg. Chloralose-urethane. Action of dibenamine to fore-quarters of recipient on reflex vasoconstriction in hind limbs.

From above: Blood pressure donor, blood pressure recipient, blood flow through the hind limbs of recipient, marks indicating occlusion of the carotids, time marking.

A) before dibenamine. B) after dibenamine.

the common carotids proximally to the carotid sinus region, or by the intravenous injection of small amounts of acetylcholine. In order to enhance the vasopressor responses the buffer action of the baroreceptors in the aortic region was eliminated by severing both vagi.

Results.

Fig. 4 illustrates an experiment of this type. Both common carotids of the recipient were clamped. This elicited a pronounced

rise in blood pressure in the upper part of the animal. Simultaneously a marked reduction of the blood flow through the hind limbs occurred as a result of the increased vasoconstrictor discharge through the abdominal sympathetic chains.

Dibenamine 20 mg/kg was then given to the forequarters, causing a fall of blood pressure to 100 mg Hg. It was regularly observed in our experiments on animals with a low blood pressure, due to bleeding or other circumstances, the vasopressor response to lowering the pressure in the carotid sinus was very weak. To obtain a blood pressure in the recipient of the same level as before dibenamine, the blood pressure was therefore raised to about the original height by small amounts of vasopressin (Hypadrin, Astra) and by transfusion of blood. After these precautions reduction of the pressure in the carotid sinus elicited a marked reduction of the blood flow through the hind limbs more than an hour after dibenamine 20 mg/kg had been given (see fig. 4 B).

As will be shown in later experiments dibenamine in the doses used completely blocks the action of vasoconstrictor impulses induced by the activation of vasoconstrictor centres. The fact that a reduced but still marked rise in blood pressure occurs in the forequarters given dibenamine, requires therefore some comment.

In the isolated fore-quarters occlusion of the common carotids excludes a considerable part of the remaining peripheral vascular bed. It seems reasonable to ascribe the remaining rise in blood pressure at least partly to haemodynamic effects. On the other hand BAYLISS (1901, 1902), BACQ *et al.* (1934) and recently BACH (1946) claim that the vasodepressor response elicited by stimulation of the depressor nerve is due to the activation of vasodilator fibres in the dorsal roots. They further claim that these fibres mediate impulses which are concerned with the maintenance of a vasodilator tone. In our experiments spinal vasodilator fibres in the upper part of the recipient are intact whilst impulses carried in such fibres do not reach the lower part. The persisting pressor response in the fore-quarters elicited by occlusion of the common carotids (fig. 4 B) is possibly to some extent due to an inhibition of a vasodilator tone. BROWN and MAYCOCK (1940) experimenting on sympathectomized cats brought evidence to indicate that there might be vasoconstrictor nerves not emanating from the thoracico-lumbar outflow running in spinal nerves. In our experiments the possible activation of such fibres can not be excluded. An additional factor raising the blood pressure might be the increase of heart rate occurring when the carotids are clamped (see table 1).

We have not attempted a detailed analysis of the pressor response remaining in the fore-quarters of the recipient under the influence of dibenamine, as this phenomenon does not interfere with the

Table 1.

Increase of Heart Rate due to Occlusion of the Common Carotids of the Recipient from fig. 5 given Dibenamine 20 mg/kg.

Heart beats per minute.	
Control	During occlusion of common carotids
210	220
214	224
208	224
214	234

validity of our observations concerning the action of dibenamine on transmission in the CNS.

The reflex vasoconstriction in the lower part of the recipient, induced by the intravenous injection of acetylcholine to the upper part, was not altered in magnitude by giving dibenamine to the upper part.

Asphyxia was produced in the recipient by occlusion of the tracheal cannula for a short period. A rise of blood pressure in the fore-quarters and a vasoconstriction in the hind limbs was observed. After dibenamine to the upper part of the recipient the same procedure evoked a vasodepression in the fore-quarters but still a vasoconstriction in the hind limbs, due to a dominant peripheral vasodilator action of CO_2 in the upper part, whilst the lower part was solely under the influence of the central action of CO_2 (see page 385).

Uniform results were obtained in all of five cross-circulation experiments with dibenamine given to the fore-quarters of the recipient. The drug did not significantly interfere with the transmission in the nervous pathways involved in eliciting reflex vasoconstriction in the hind limbs.

C. Action of Dibenamine on the Neuro-Effector Transmission at Accelerant Nerve Endings.

As reported above an immediate increase in the heart rate occurred when the common carotids were clamped in a cat given dibenamine (table 1). The fact that dibenamine does not block the release of the mediator at the adrenergic nerve endings to the heart, was confirmed in experiments where the accelerant nerves were stimulated.

In three artificially respiration cats a Sherrington electrode was inserted through a hole in the chest between the second

and third ribs and placed around the right stellate ganglion. The sympathetic trunk above the ganglion was cut. The ganglion and the cardiac nerves emanating from it were stimulated by electrical stimuli. The cardiac acceleration induced by the stimulation was not reduced by dibenamine. This is shown in table 2.

Table 2.

Effect of Stimulation of Right Stellate Ganglion on Heart Rate.

I) Cat 2.7 kg. Chloralose-urethane.

Heart beats per minute.

Before stim.	During stim.	Increase in %	
200	236	18	
190	224	18	
186	224	20	mean value
192	214	11	16 %
192	228	19	
196	216	10	
One hour after dibenamine 20 mg/kg			
172	212	23	
172	192	12	
188	216	15	mean value
184	204	11	12.3 %
178	196	10	

II) Cat 2.5 kg. Chloralose-urethane.

Before stim.	During stim.	Increase in %	
234	272	16	
236	272	15	mean value
252	266	6	12.3 %
One hour after dibenamine 20 mg/kg			
220	266	21	
218	250	14	
220	270	23	mean value
232	250	16	18.2 %
236	276	17	

III) Cat 2.5 kg. Chloralose-urethane.

Before stim.	During stim.	Increase in %	
230	280	22	
240	278	16	
232	276	19	mean value
228	270	18	18.8 %
One hour after dibenamine 20 mg/kg			
188	248	32	
210	258	23	mean value
212	260	23	26 %

Comments.

In part II of this paper it is demonstrated that dibenamine does not significantly interfere with the transmission of impulses in central and peripheral nervous structures involved in producing reflex vasoconstrictor discharges to the hind limbs. The drug does not block the neuro-effector transmission at the junction of adrenergic nerve endings and the heart. NICKERSON and GOODMAN observed an acceleration of the heart when stimulating the left splanchnic nerves on adrenalectomized cats given dibenamine. They ascribe these effects to the remote action of released sympathin E. In its biological action dibenamine thus seems to be closely related to ergotamine. The most likely explanation of its action is that it blocks the excitatory effects of the transmitter at adrenergic nerve endings without interfering with the release of this transmitter.

PART III.

Action of Dibenamine and Ergotamine on Vasoconstrictor Responses due to Reflex Activation of Vasoconstrictor Centres.

A. Vessels of the Hind Limbs.

Experimental.

Cross-circulation with two cats was arranged as described on page 7 (see fig. 3). It will be recalled that in this arrangement all vascular connections between the upper and lower parts of the recipient cat were blocked and that the intact abdominal sympathetic chains constituted the only nervous connection between these parts.

Vasoconstrictor discharges to the hind limbs of the recipient were produced by clamping the common carotids proximally to the carotid sinus region, by intravenous injection of acetylcholine, by asphyxia, by inhalation of CO₂ or by stimulation of the central stump of the brachial nerve.

Fig. 5 shows a typical experiment of this type. Intraarterial injections of 1 γ adrenaline, and 1 γ nor-adrenaline into the inferior mesenteric artery cause a vasoconstriction with a decreased blood flow through the hind limbs.

Clamping both common carotids of the recipient elicits a pronounced blood pressure rise in the fore-quarters and a marked

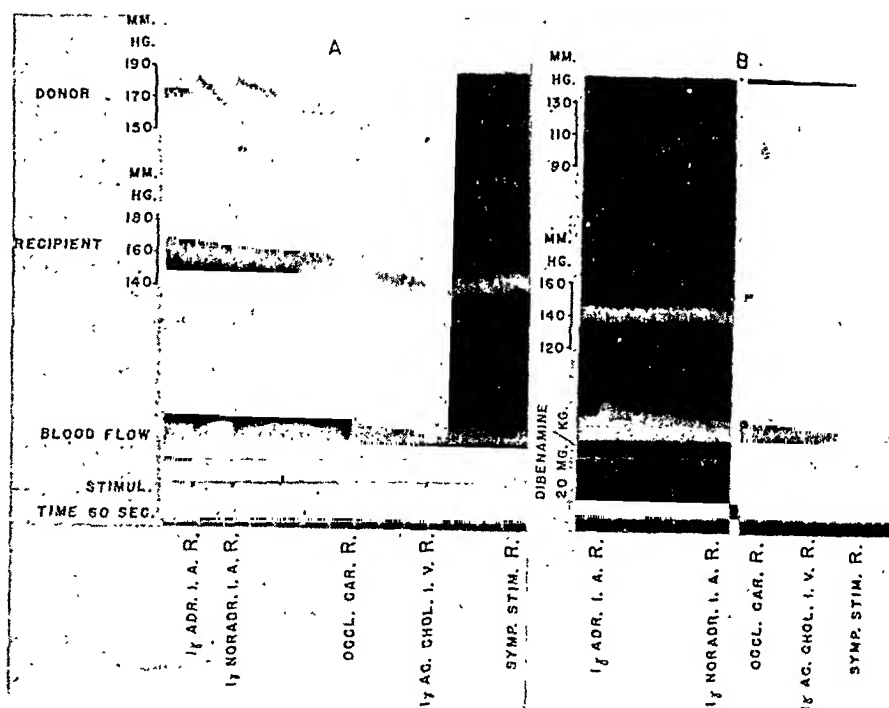


Fig. 5. Cross-circulation. Donor 2.7 kg. Recipient 2.5 kg. Chloralose-urethane. Action of dibenamine to hind limbs of recipient on vasoconstrictor responses to adrenaline, nor-adrenaline, and reflex vasoconstrictor impulses.

From above: Blood pressure donor, blood pressure recipient, blood flow through hind limbs of recipient, stimulation and injection marks, time marking.

A) before dibenamine. B) after dibenamine 20 mg/kg.

vasoconstriction in the hind limbs. The increased peripheral resistance in the perfused hind limbs induces a blood pressure rise in the donor.

Injection of acetylcholine into the brachial vein of the fore-quarters elicits an abrupt blood pressure fall and an outflow of vasoconstrictor impulses to the hind limbs analogous with the events on occlusion of the carotids. Note the secondary blood pressure rise in the donor.

Stimulation of the abdominal sympathetic chain at L 5 results in a vasoconstriction in the hind limbs with a consequent blood pressure rise in the donor.

Dibenamine is now given to the donor. Fig. 5 shows the picture 60 minutes after dibenamine 20 mg/kg was given.

1 γ adrenaline elicits a marked vasodilatation whilst the vasoconstrictor response to nor-adrenaline is completely abolished. Although the vascular responses in the upper half of the recipient

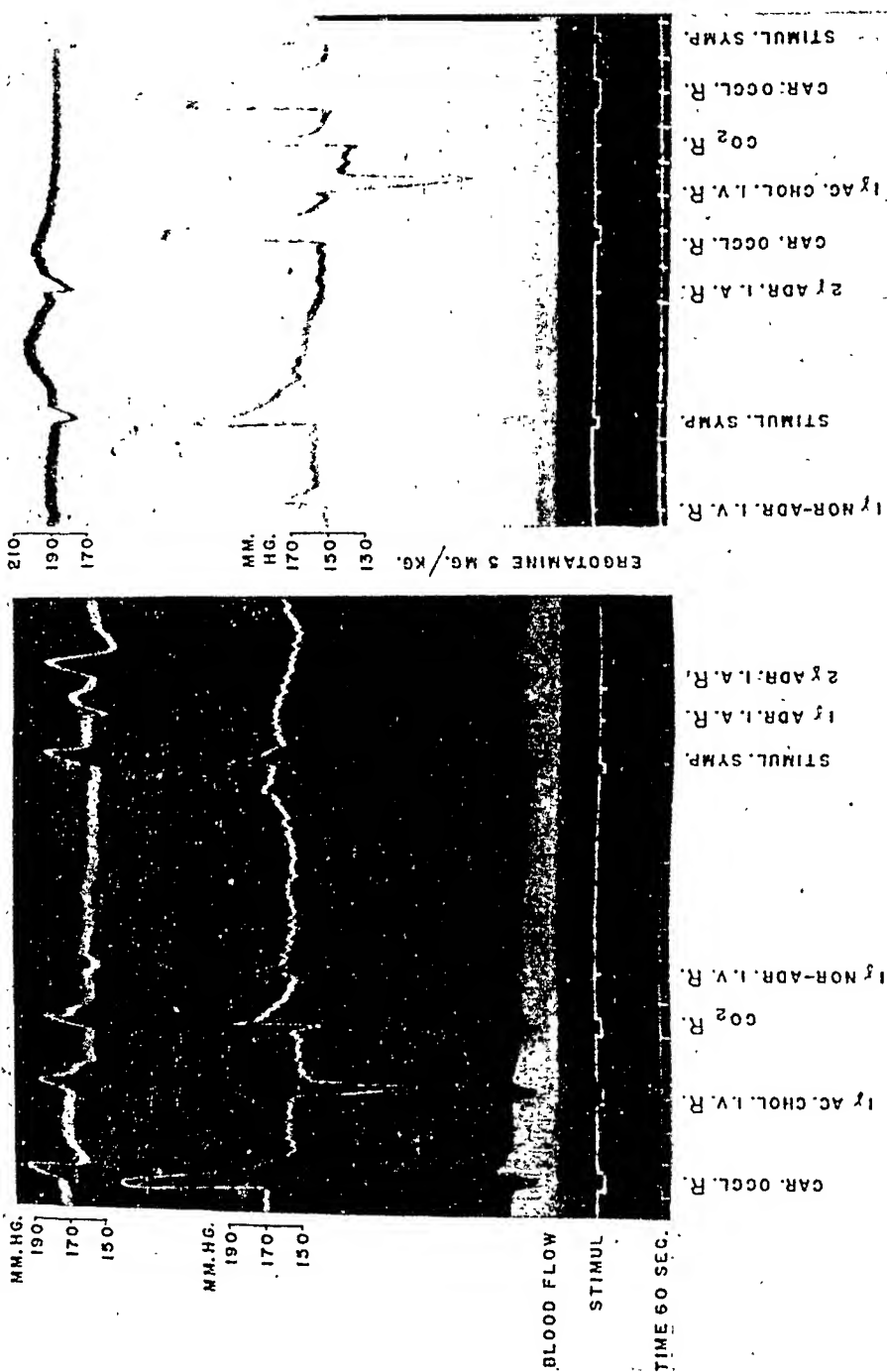


Fig. 6. Cross-circulation. Donor 1.8 kg. Recipient 2.8 kg. Chloralose-urethane. Action of ergotamine to hind limbs of recipient on vasoconstrictor responses to adrenaline, nor-adrenaline, and reflex vasoconstrictor impulses. From above: Blood pressure donor, blood pressure recipient, blood flow through the hind limbs of the recipient, stimulation and injection marks, time 60 seconds.
A) before ergotamine. B) after ergotamine 5 mg/kg.

elicited by clamping of the carotids, or by intravenous injection of acetylcholine remain unchanged in magnitude, *the concomitant vasoconstriction in the hind limbs completely disappears*. On the other hand the vasoconstrictor response to sympathetic stimulation is reversed.

9 cross-circulation experiments of this kind gave quite uniform results. However, as dibenamine is a new drug, as yet little studied for the sake of comparison some experiments were performed with ergotamine. The results were indetical with those obtained with dibenamine. One experiment is shown in fig. 8, presented as it shows some facts not seen in fig. 7. Ergotamine, 5 mg per kg, given slowly intravenously completely abolishes reflex vasoconstriction in the hind limbs and the vasoconstrictor response to nor-adrenaline. The vasoconstrictor responses to stimulation of the sympathetic chain and to adrenaline are reversed. A vasodilatation occurs in the hind limbs of the recipient followed by a fall of blood pressure in the donor. — Note the definite blood pressure variations in the donor cat following the vasomotor responses in the hind limbs.

In this experiment 1 γ nor-adrenaline was given *intravenously* to the recipient. The consequent blood pressure rise in the fore-quarters evokes a slight vasodilatation in the hind limbs and a consequent fall of blood pressure in the donor. After ergotamine the same blood pressure rise in the fore-quarters does not influence the blood flow through the hind limbs (see FOLKOW and UVNÄS 1948).

The blood pressure rise in the fore-quarters as the result of stimulation of the sympathetic chains (see *e. g.* fig. 6) evidently is due to stimulation of afferent fibres. This pressor response frequently observed in the fore-quarters persisted after dibenamine or ergotamine was given to the donor but disappeared completely after dibenamine was given to the fore-quarters.

According to BURN (1938) the vasoconstriction which occurs on stimulation of the sympathetic chain of a dog is chiefly in the skin. He considers about 20 per cent of the reduction of the leg volume to be due to constriction in the muscles. Our observations on cats with skinned or unskinned legs do not agree with those of Burn on dogs. In the cat the major part of the reduction of blood flow on stimulation of the sympathetics is due to vasoconstriction in the muscles. However, to satisfy ourselves that the vasoconstriction elicited by activation of vasomotor centres

was not mainly restricted to the skin we made a cross circulation experiment with the hind limbs of the recipient skinned. In this experiment clamping the common carotids or injection of acetylcholine intravenously still elicited a pronounced vasoconstriction in the hind limbs. As was the case in experiments with unskinned legs the vasoconstriction was annulled but not reversed after dibenamine.

Among recent investigators BIÖRCK (1947) and GERNANDT and ZOTTERMAN (1946) on cats given dibenamine (B) or ergotamine (G and Z) observed a vasodepressor effect with the inhalation of CO_2 . G and Z ascribe this depressor action of CO_2 mainly to the peripheral action of accumulated CO_2 , partly to the reversed effect of adrenaline liberated at adrenergic nerve endings or to the action of sympathin I.

In a few experiments we exposed the recipient to an increased CO_2 content in the inhaled air. As seen in fig. 6 this procedure caused a vasopressor effect in the fore-quarters as well as in the hind limbs. — Note the consequent blood pressure rise in the donor. — After ergotamine 5 mg/kg (or dibenamine 20 mg/kg) to the donor the vasoconstrictor response in the hind limbs was *completely annulled but not reversed* although the pressor response remained unaffected in the fore-quarters. Similar results were obtained when asphyxia was produced in the recipient by obstructing the tracheal cannula for a short period. Dibenamine or ergotamine given to the donor *annulled but did not reverse* the vasoconstrictor response in the hind limbs.

In some other experiments, dibenamine, 20 mg/kg, was given to the recipient (fore-quarters). Before dibenamine asphyxia of the recipient elicited a pressor response but, as seen in fig. 7, after dibenamine a pronounced vasodepression occurred in the fore-quarters. The persistent constrictor discharge to the hind limbs, however, still caused vasoconstriction there. If asphyxia was then produced in the donor, a vasopressor response occurred in that animal, demonstrated by a marked blood pressure rise. Concurrently a pronounced vasodilatation occurred in the *perfused hind limbs*. Since only *vascular* connections existed between the two animals the increased blood flow in the hind limbs must have been due to the combined action of local anoxia and the increased CO_2 -tension in the blood. Identical results were observed when the donor was exposed to an increased CO_2 concentration in the inhaled air.

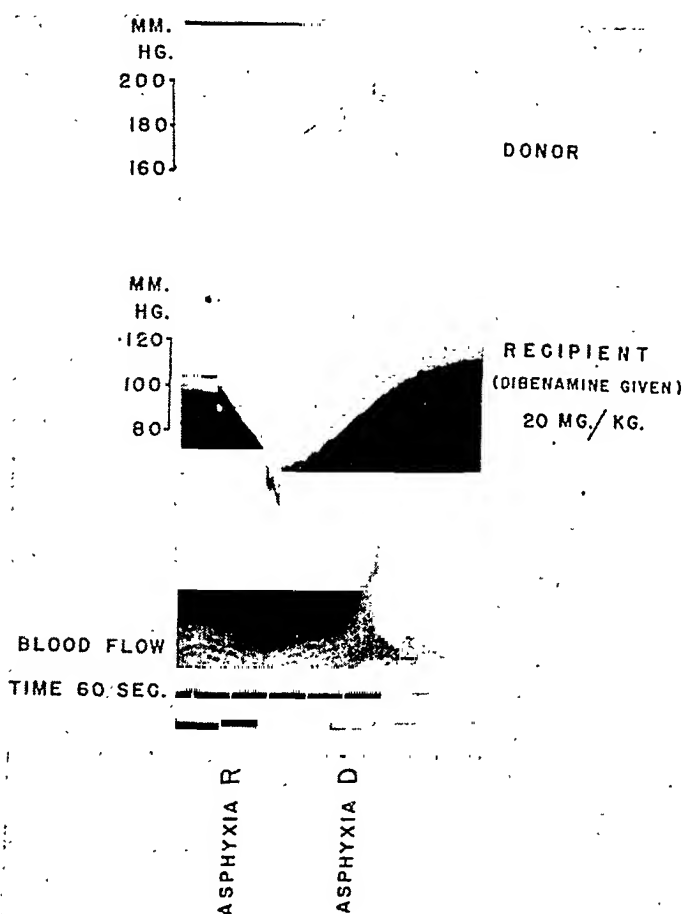


Fig. 7. Cross-circulation. Donor 3 kg. Recipient 2.6 kg. Chloralose-urethane. Effect of asphyxia of donor or recipient on blood flow and blood pressure.

From above: Blood pressure donor, blood pressure recipient, blood flow from hind limbs of recipient, time marking.

B. Vessels of the Splanchnic Region.

The action of dibenamine on reflex vasoconstriction in the splanchnic region was recorded in three cross-circulation experiments. Cross-circulation was arranged between two cats as follows.

The donor cat was eviscerated as described on page 367. The abdomen of the recipient was opened by a midline incision and the spleen removed. The superior mesenteric artery was dissected free at its proximal end and cannulated, care being taken not to damage the adjacent nerves. Cranial and caudal vascular connections from the splanchnic region were obstructed by ligatures around the inferior mesenteric artery, the rectum and the proximal end of the duodenum. Finally the portal vein was prepared free, ligated and cannulated

distally to the ligature. Both animals were heparinized and cross-circulation established. By rubber tubings directed through stab wounds in the abdominal wall the proximal end of the aorta of the eviscerated donor was connected with the superior mesenteric artery of the recipient, and the portal vein of the recipient with a Gaddum recorder. From the recorder the blood was drained to the proximal part of the ligated lower caval vein of the donor. By this arrangement the splanchnic region of the recipient received its blood supply from the donor whilst its vasomotor control was supplied via the intact sympathetic nerves. Adrenaline and nor-adrenaline were administered by "close intraarterial" injections into the rubber tubing leading to the superior mesenteric artery. The blood pressure of the recipient was recorded in the brachial artery.

Results.

Intraarterial injections of adrenaline, or nor-adrenaline regularly elicited a vasoconstriction of the splanchnic vessels. Clamping of the common carotids of the recipient below the carotid sinus regions, or injection of acetylcholine into the brachial vein of the recipient elicited a pronounced reflex vasoconstriction of the splanchnic vessels. Under the influence of dibenamine 20 mg/kg the vasoconstrictor response to adrenaline was regularly reversed to a vasodilatation, although this was not as marked as in the muscular vessels. The constrictor actions of nor-adrenaline or reflex vasoconstrictor impulses were *more or less annulled but never reversed*. Fig. 8 illustrates one of these experiments.

Comments.

As appears from the papers cited on the pages 365—367, there is contradictory evidence to the rather generally accepted conception that adrenaline is the transmitter at sympathetic vasoconstrictor nerve endings. In our cross-circulation experiments under the influence of dibenamine and ergotamine in adequate doses, stimulation of the abdominal sympathetic chains or injection of adrenaline causes vasodilatation contrasting with constriction in the untreated animal. When, however, exclusively vasoconstrictor fibres are activated by reflex stimulation of vasoconstrictor centres the vasoconstrictor response is, under the influence of dibenamine or ergotamine *reduced or annulled but not reversed*. Since there is no indication that dibenamine or ergotamine interfere with the release of the adrenergic transmitter our experiments suggest that this transmitter is not adrenaline; to comply with our observations the transmitter should lack vasodilator properties. From our observations nor-adrenaline fulfills this requirement;

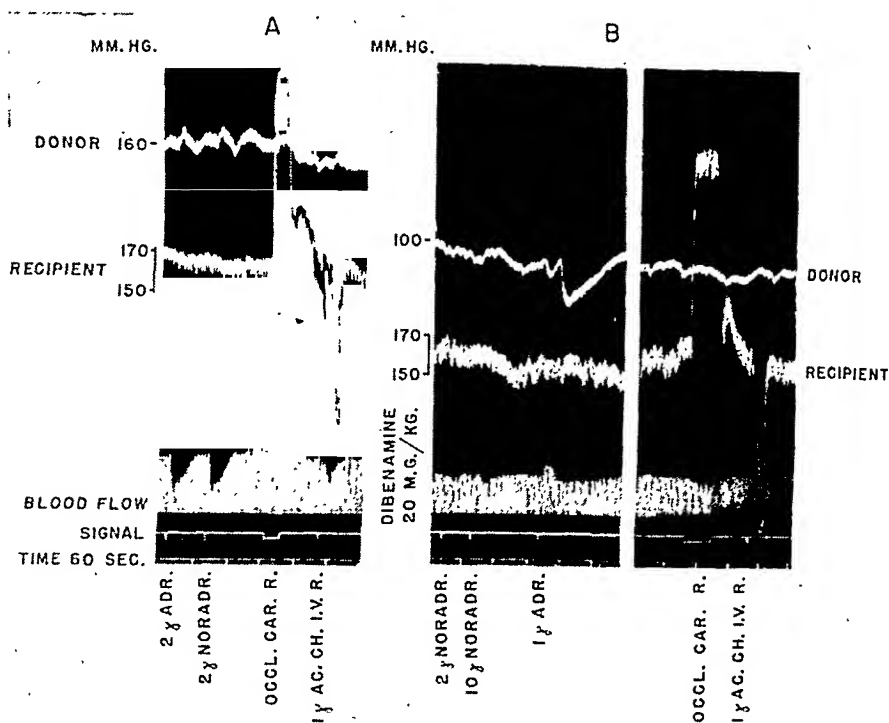


Fig. 8. Cross-circulation. Donor (D) 3.2 kg. Recipient (R) 2.8 kg. Chloralose-urethane. Action of dibenamine on constrictor responses in splanchnic region elicited by adrenaline, nor-adrenaline and reflex constrictor impulses.

From above: Blood pressure donor, blood pressure recipient, blood flow in splanchnic region, marks indicating occlusion of carotids, and injection of drugs, time marking.

A) before dibenamine. B) after dibenamine (when adrenaline is given. Note the initial small fall of blood pressure concomitantly with the increase of blood flow followed by a marked vasodepression caused by the appearance of adrenaline in the general circulation).

under the influence of ergotamine or dibenamine its vasoconstrictor effect is reduced or annulled and it does not cause vasodilatation. Our results are in full agreement with the view held by EULER that nor-adrenaline actually might be the adrenergic transmitter.

The vasodilatation produced by stimulating the abdominal sympathetic chains seems to us to be adequately explained by the activation of sympathetic vasodilators. This assumption agrees well with observations of DALE (1913), CANNON and ROSENBLUETH (1935), BÜLBRING and BURN (1935) and others. In following papers we present further evidence for the existence of efferent sympathetic vasodilators, cholinergic in their mode of action, in the hind limbs of the cat.

Summary.

Experiments are presented which indicate that dibenamine blocks the effector cells to the vasoconstrictor action of the adrenergic transmitter and adrenaline and that it does not interfere with the release of the adrenergic transmitter.

The transmitter at vasoconstrictor nerve endings in the cat is not likely to be adrenaline; to satisfy our observations the transmitter should be a substance with exclusively vasoconstrictor action. It might be nor-adrenaline.

The vasodilator response to stimulation of the sympathetic chains after dibenamine is adequately explained as due to activation of vasodilator fibres. The vasodepressor action caused by CO₂ or asphyxia after dibenamine or ergotamine is due to the peripheral action of CO₂ and metabolites.

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From the department of Physiology, University of Lund.

The Distribution and Functional Significance of Sympathetic Vasodilators to the Hind Limbs of the Cat.

By

BJÖRN FOLKOW and BÖRJE UVNÄS.

Received 5 March, 1948.

In a previous paper from this laboratory (FOLKOW and UVNÄS 1948) we reported that dibenamine and ergotamine annul the vasoconstriction in the hind limbs of the cat elicited by reflex activation of vasoconstrictor centres. On the other hand, under the influence of dibenamine or ergotamine, stimulation of the abdominal sympathetic chains resulted in a pronounced vasodilatation interpreted by us as indicating the activation of vasodilator fibres. As very little is known about the distribution and functional significance of sympathetic vasodilators in the cat an investigation on this subject was carried out.¹

Experimental.

When not otherwise stated the experimental procedure was the same as reported by FOLKOW and UVNÄS (1948). The blood flow in the partly isolated hind limbs of the cat was measured by a Gaddum recorder (GADDUM 1929). The sympathetic trunks, cut at the height of L₂ and dissected free down to the ganglion of L₅, were stimulated electrically. The blood pressure was recorded in the brachial artery by a mercury manometer. Dibenamine, when used, was given slowly intravenously.

¹ For papers dealing with sympathetic vasodilator fibres in the dog and cat see FOLKOW, HÆGER and UVNÄS (1948).

a) *Selective stimulation of vasodilator fibres.*

In 9 experiments the sympathetic trunks were stimulated by square shaped, sinus shaped and spike shaped stimuli, and frequency, duration and intensity varied.

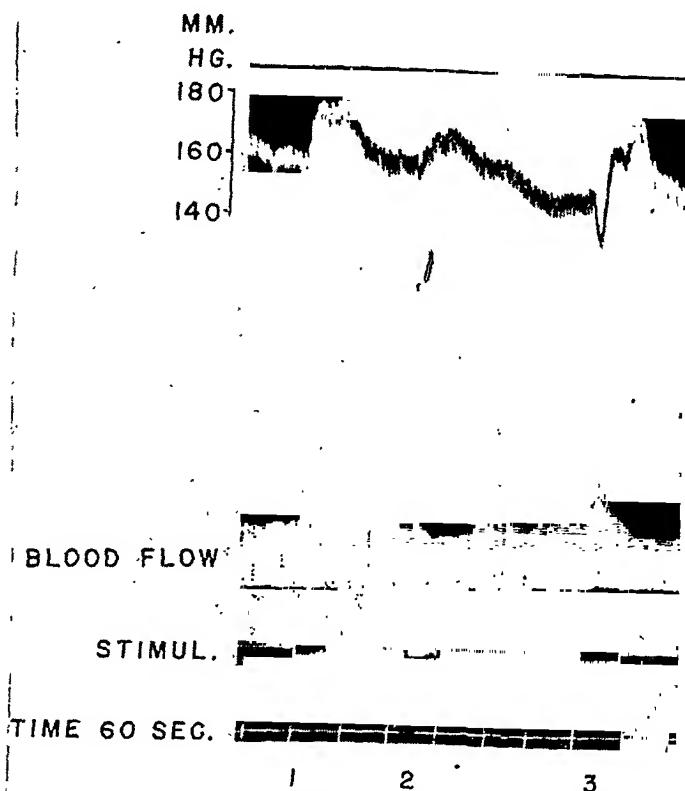


Fig. 1. Cat 3.3 kg. Chloralose-urethane. Vasoconstrictor and vasodilator responses to stimuli of various frequency and strength.

From above: Blood pressure, blood flow through the hind limbs, stimulation marks.

1) Stim. 30 sec. 3 V. frequency 60/sec. 2) Stim. 30 sec. 2 V. frequency 60/sec. 3) Stim. 60 sec. 3V. frequency 15/sec.

In three of these experiments significant vasodilator effects were observed. A dilator response of this type is shown in fig. 1 where spike shaped stimuli were used. Stimuli with a frequency of 60/sec. induced a marked vasoconstriction (1 and 2 in fig. 1). When the frequency was changed to 15/sec. an initial dilatation, followed by a constriction occurred (3 in fig. 1). — Note the concomitant increase of blood flow and fall of blood pressure. — In the second experiment in which vasodilator responses were ob-

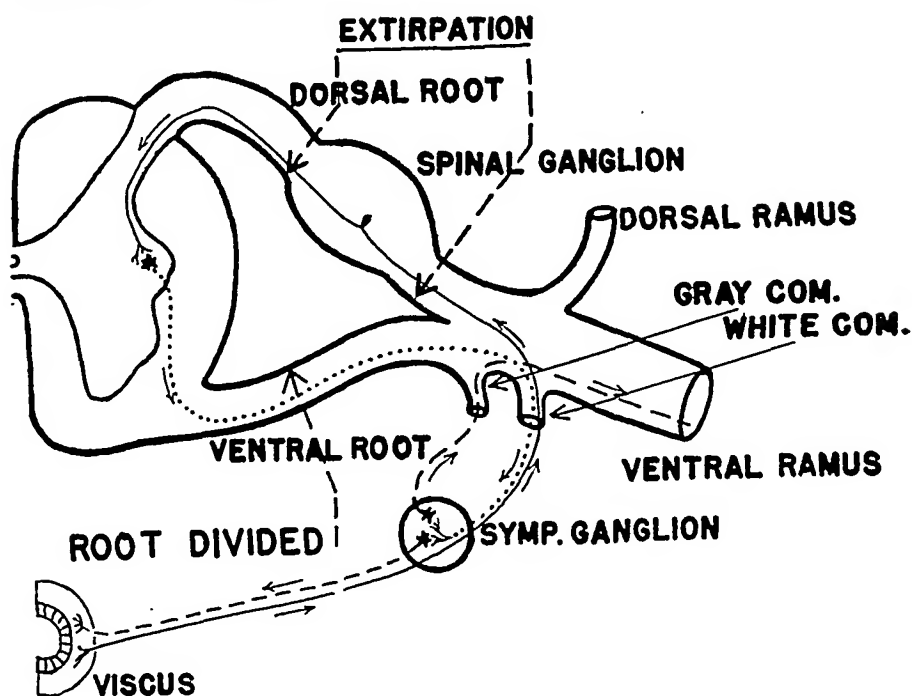


Fig. 2. Diagrammatic section through a spinal nerve and the spinal cord to illustrate the chief functional types of peripheral nerve fibres (from RANSON, *Anatomy of the Nervous System* 1939). The points of ganglionectomy and anterior root section are indicated.

served square formed stimuli were used and sinus formed in the third.

In order to investigate whether the vasodilatation was due to the stimulation of afferent or efferent fibres, two groups, each of 3 cats, were operated on as described below.

b) *Elimination of afferent fibres.*

The anatomical arrangement of the thoracico-lumbar division of the autonomic nervous system of the cat makes it possible to sever the dorsal roots peripherally to the ganglion without damaging the efferent sympathetic outflow in the spinal nerves. The grey communicant fibres join the spinal nerve fibres a few mm peripherally to the spinal ganglion (see fig. 2). The efferent sympathetic fibres to the hind limbs of the cat leave the spinal column through the four upper lumbar anterior roots. We have not been able to find out from the available literature to what extent afferent fibres from the hind limbs pass through the sympathetic chain at the level stimulated in our experiments. But, if there are such fibres at this level, the majority of them probably enter

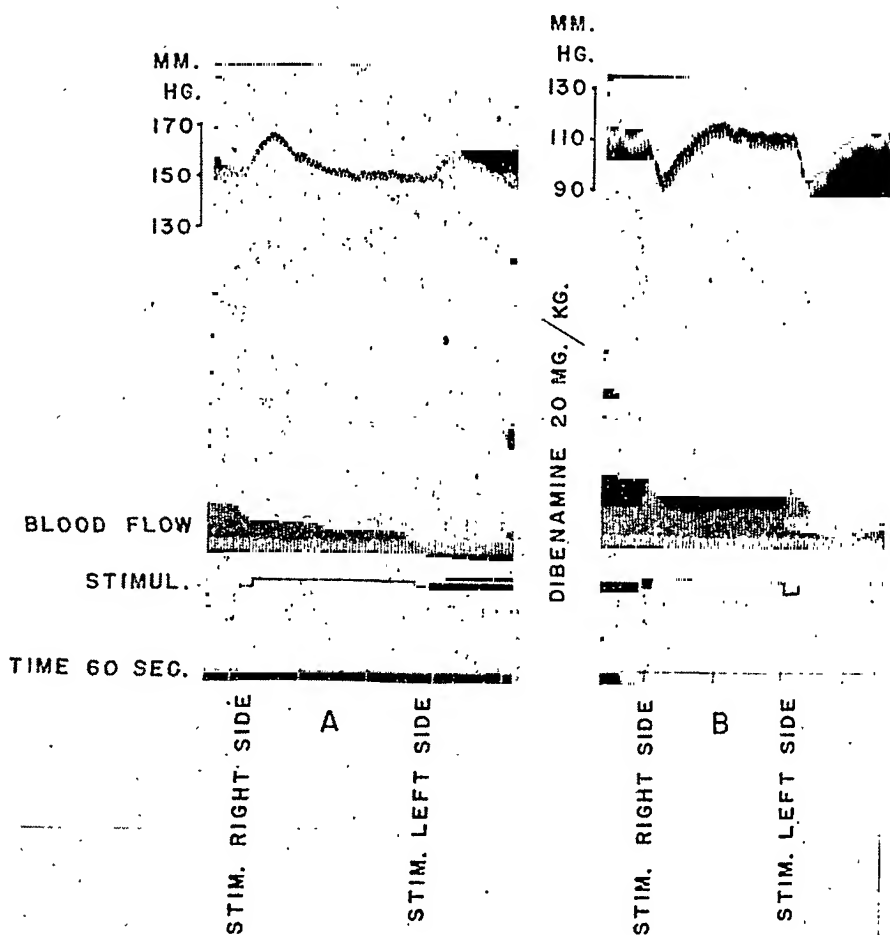


Fig. 3. Cat 3.4 kg. Chloralose-urethane. Spinal ganglia L_1-L_5 extirpated 14 days previously to the experiment.

From above: Blood pressure, blood flow through the hind limbs, stimulation marks, time marking.

A) Vasoconstrictor responses to the separate stimulation of the right and left sympathetic chain.

B) After dibenamine. Vasodilator responses to the separate stimulation of the right and left sympathetic chain.

the spinal medulla through the dorsal roots of the segment in which the sympathetic outflow to the hind limbs is found.¹

After extirpation of the spinal ganglia L_1-L_5 and consequent degeneration of the dorsal root fibres, vasodilator responses to the stimulation of the sympathetic trunks, if due to antidromic stimulation, should disappear or diminish.

¹ A schematic drawing illustrating the sympathetic outflow to the hind limbs of the cat is presented in a previous paper (Folkow and Uvnäs 1948 Fig. 2).

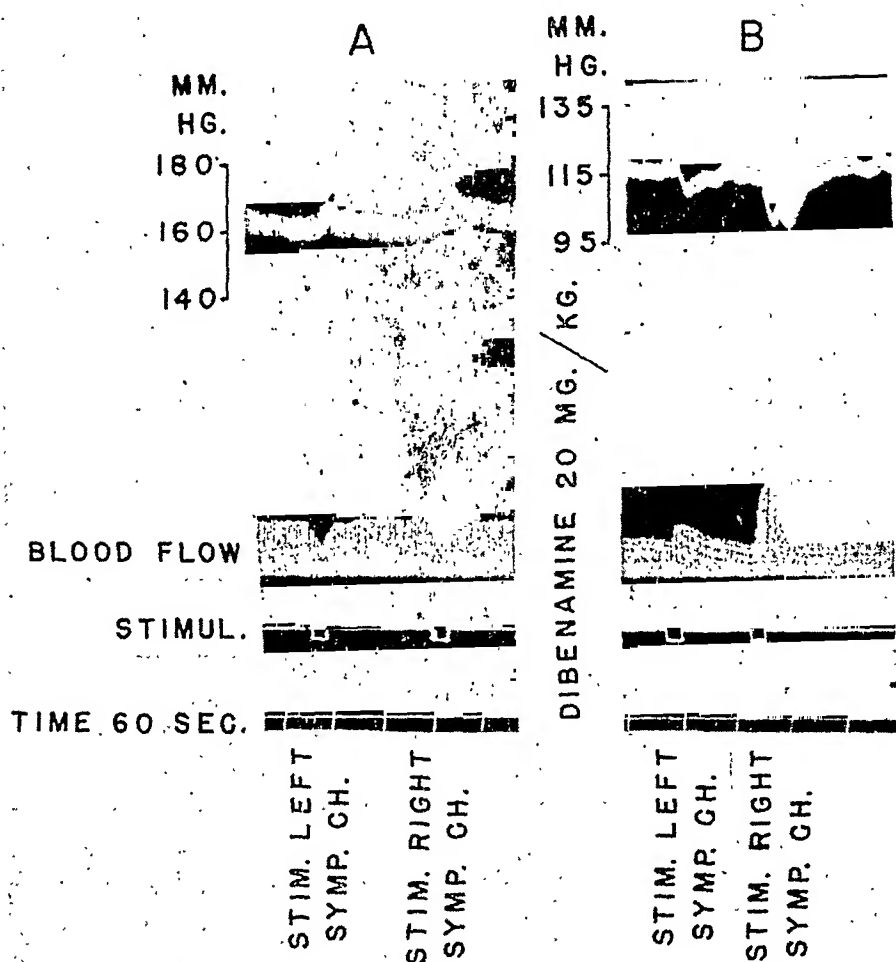


Fig. 4. Cat 3 kg. Chloralose-urethane. The anterior roots of L_1-L_5 sectioned 11 days prior to the experiment.

From above: Blood pressure, blood flow through the hind limbs, stimulation marks, time marking.

- A) Vasoconstrictor responses to the separate stimulation of the left and right sympathetic chain.
 B) After dibenamine. Vasodilator responses to the separate stimulation of the left and right sympathetic chain.

With these premises we severed the left dorsal roots of L_1-L_5 in the following way. Under nembutal anaesthesia and sterile conditions the vertebral arches of the five upper lumbar vertebrae were exposed on the left side. With a dentist's drill an opening was made over a dorsal root with its ganglion. This was isolated from the anterior root with careful dissection, and by cutting the dorsal root just proximally and distally to the ganglion it was prepared free and removed. By the same procedure the five upper lumbar spinal ganglia were extirpated, leaving the sympathetic outflow to the hind limbs intact.

The dorsal root fibres were then allowed to degenerate. After 10—14 days the animals were used in acute experiments. The experimental procedure was the same as described by Folkow and Uvnäs 1948, the only difference being that the two sympathetic chains were stimulated separately by separate Collison electrodes.

Fig. 3 shows the observations made on one of these animals, the spinal ganglia of which had been removed 14 days previously. The sympathetic chains were stimulated separately by stimuli of identical intensity and duration. There was no significant difference in the vasoconstrictor responses obtained on stimulation of the right and left sympathetic chain indicating that the efferent sympathetic outflow had not been perceptibly damaged by the operative procedure. After dibenamine 20 mg/kg the dilator response to stimulation of the sympathetic chain was of the same magnitude on both sides.

c) *Elimination of efferent sympathetic fibres.*

With the technique we employed pre- as well as postganglionic fibres of the sympathetic chain are included in the electrode. For this reason the vasomotor response to electrical stimulation of the chain cannot be abolished but merely reduced by section and consequent degeneration of preganglionic fibres.

On the second group of cats the spinal roots of L_1 — L_5 were exposed as described under b, and the corresponding anterior roots cut. After 10—14 days the animals were used in acute experiments. The experimental procedure was as described under b.

Before dibenamine, stimulation of the left sympathetic chain elicited a weaker vasoconstriction than stimulation on the unoperated side. After dibenamine the vasodilator response was considerably weaker on the operated side (fig. 4).

Distribution of the Vasodilators.

a) *Experiments on skinned limbs.*

The magnitude of the vasodilator responses observed indicated that the dilator fibres were distributed mainly to the muscles of the legs. This assumption was confirmed in three experiments on skinned limbs. Fig. 5 represents one of these experiments. The animal was given dibenamine 15 mg/kg. Stimulation of the sympathetic chains elicited a marked dilator response. The hind legs were then skinned, the tail, and the extremities amputated distally

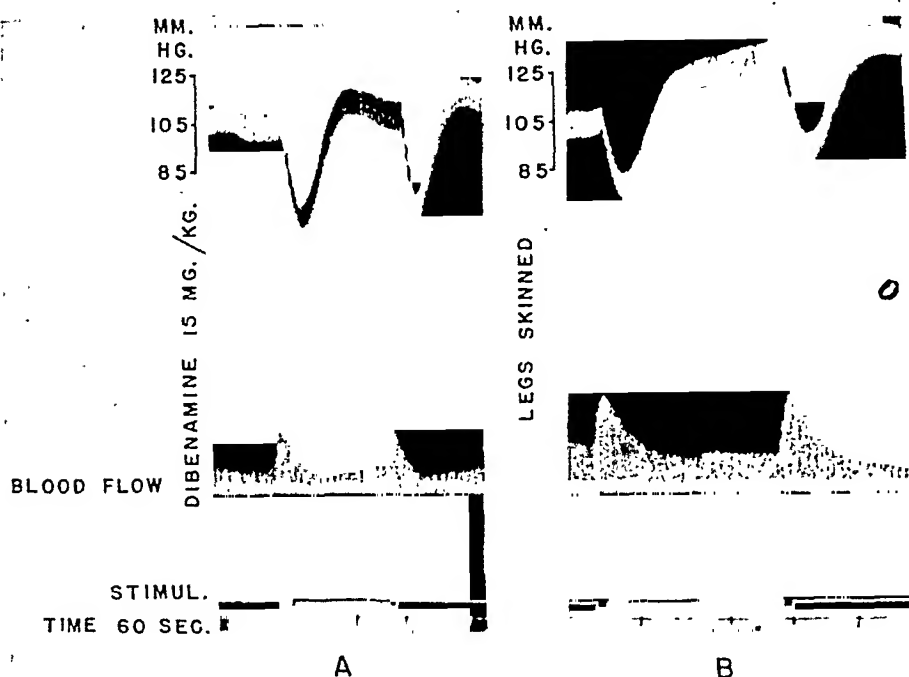


Fig. 5. Cat 3.3 kg. Chloralose-urethane. Dibenamine 15 mg/kg given.

From above: Blood pressure, blood flow through the hind limbs, stimulation marks, time marking.

- A) Twice repeated stimulation of the sympathetic trunks.
B) The same procedure with the limbs skinned.

to the ankle joints. A marked vasodepressor response was still observed when the sympathetic trunks were stimulated.

b) *Simultaneous observations on vasomotor responses in muscles and skin.*

In order to obtain more precise information concerning the distribution of the sympathetic dilator fibres the rate of flow was recorded individually from the caval vein and the saphenal veins. The saphenal veins were exposed, cannulated 2—3 cm below the knees, the blood flow from both sides collected and directed to a drop recorder. The volume of the blood flow from the skin was calculated from the number of drops registered per minute.

Fig. 6 represents one of these experiments. Stimulation of the sympathetic trunks caused a pronounced vasoconstriction with a decrease of the blood flow from the caval vein as well as from the saphenal veins. Dibenamine 20 mg/kg was given. 15 minutes later the vasoconstriction in the muscles on stimulation of the sympathetics changed to a vasodilatation. A marked increase in caval outflow and a fall of blood pressure occurred. In the

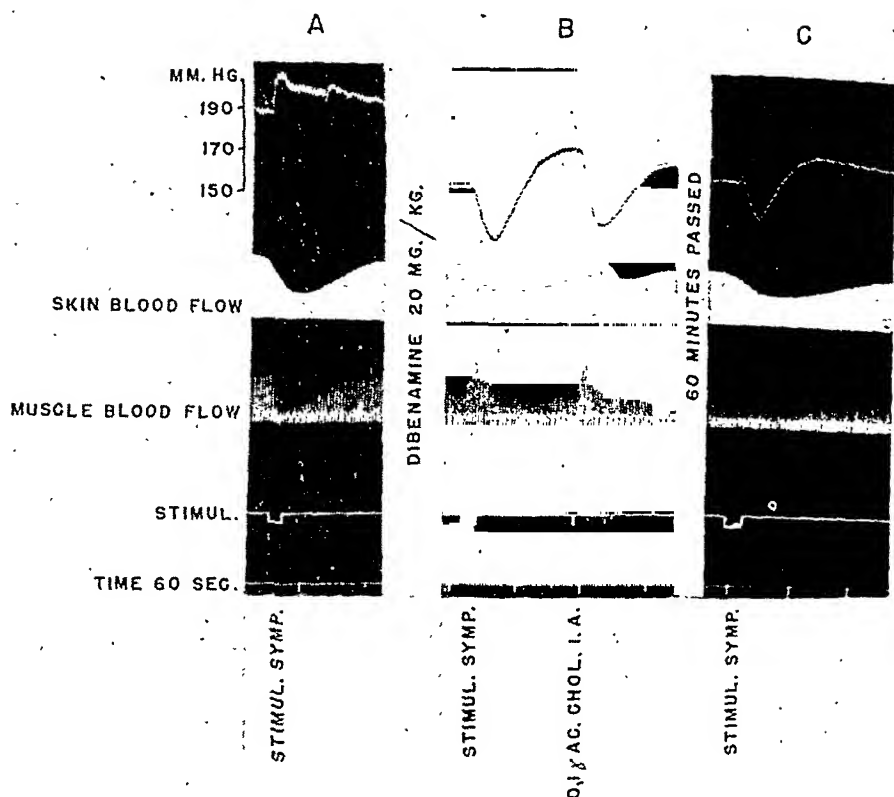


Fig. 6. Cat 2.8 kg. Chloralose-urethane. Vasomotor responses in skin and muscles to stimulation of the sympathetic chain.

From above: Blood pressure, outflow from saphenal veins, outflow from caval vein, injection and stimulation marks, time marking.

A) Before dibenamine 20 mg/kg.

B) Immediately after dibenamine.

C) 60 minutes after dibenamine.

skin the rate of flow was still diminished. 60 minutes later the picture was unaltered. Uniform results were obtained in all six experiments of this kind. Dibenamine in doses which completely reversed the sympathetic vasoconstrictor response in the muscles had no such effect in the skin. The vasoconstrictor response to sympathetic stimulation was in the skin largely but not completely blocked.

Functional Significance of the Sympathetic Vasodilators.

In cross-circulation experiments we made an attempt to obtain some information about the functional significance of the sympathetic vasodilators.

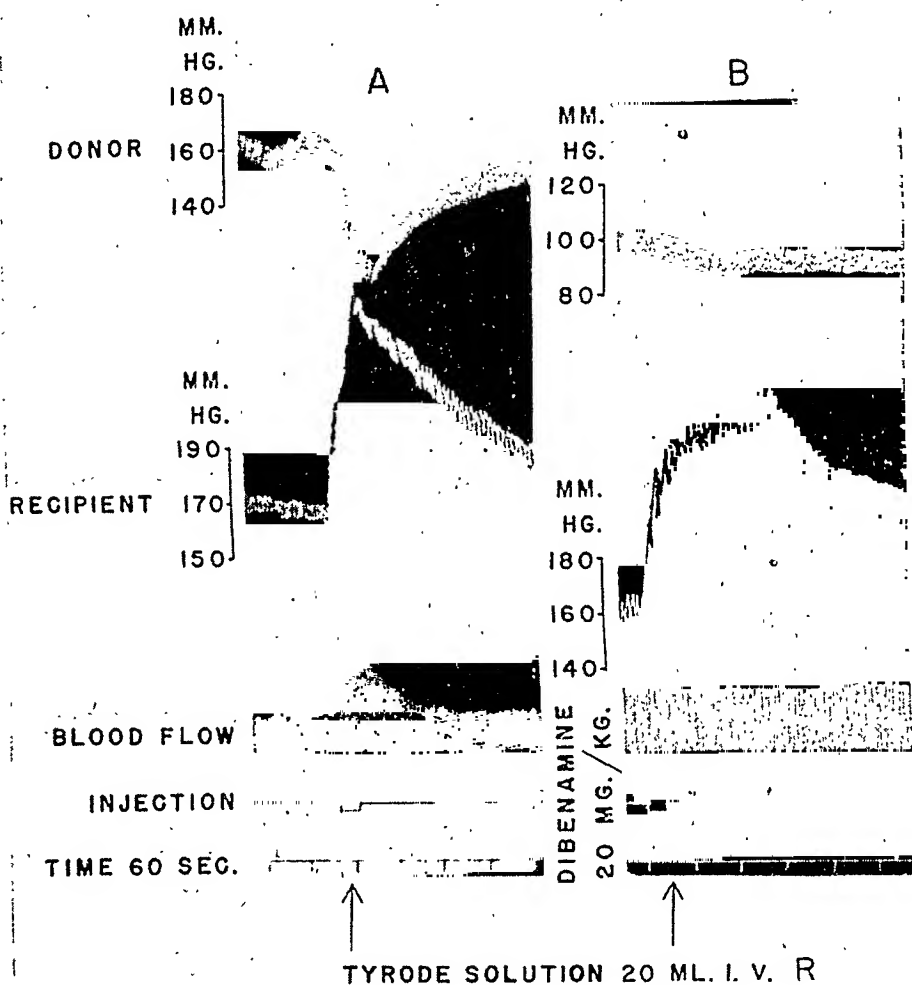


Fig. 7. Cross-circulation. Donor 3.8 kg. Recipient 4 kg. Chloralose-urethane. Vasodilator response in the hind limbs of the recipient to intravenous injection of 20 ml Tyrode's solution to the fore-quarters of the recipient. A) Before dibenamine. B) After dibenamine 20 mg/kg to the donor. Note the disappearance of vasodilatation in hind limbs.

The experimental procedure was the same as described in a previous paper by FOLKOW and UVNÄS (1948). In principle the arrangement was as follows. In one cat the hind limbs were isolated in such a way that no vascular connections existed with the upper part of the animal, and the abdominal sympathetic chains formed the only nervous connection to the CNS. The isolated hind-limbs were supplied with blood from a donor cat and the blood flow recorded by the technique described by GADDUM (1929).

A reflex vasodilatation was elicited in the hind limbs by injecting nor-adrenaline intravenously to the upper part of the reci-

pient. The blood pressure rise in this part of the animal induced a vasodilatation in the isolated hind limbs (see FOLKOW and UVNÄS 1948 fig. 6). In other experiments the blood pressure was abruptly raised in the fore-quarters of the recipient by intravenous infusion of blood or physiological saline. As seen in fig. 7 the blood pressure rise in the upper half of the recipient elicits a marked vasodilatation in the hind limbs and a concomitant blood pressure fall in the donor. After dibenamine was given to the donor no significant reflex vasodilatation could be elicited in the hind limbs. Stimulation of the abdominal sympathetic chains as usual caused a marked vasodilatation.

Stimulation of the central stump of the brachial nerve of the recipient with weak stimuli of low frequency elicited a vasodilatation in the hind limbs. This vasodilatation could not be obtained after dibenamine was given to the donor.

Comments.

The experiments show that in the cat sympathetic vasodilator fibres are distributed to the muscles of the hind legs. The fact that the vasodilator response to stimulation of the sympathetic chains is not affected by degeneration of the dorsal root fibres L_1 — L_5 , but definitely reduced after degeneration of preganglionic fibres in the sympathetic chain, in our view affords suggestive evidence that these vasodilators are efferent.

With the possible exception of the ear BÜLBRING and BURN (1936) deny the existence of sympathetic vasodilators to the skin in the dog. They found in perfusion experiments that sympathetic stimulation caused a vasoconstriction in the skin of the hind limbs of the dog. This vasoconstriction could be annulled, but never reversed by ergotoxine.

Our experiments indicate similar conditions in the cat. However, it is hard to draw decisive conclusions from our observations. As there are intimate vascular connections between the muscles and the skin haemodynamic changes in blood channels of the muscles might affect the blood flow in the skin. It is hard to predict in which direction this influence will be. Will for instance the depressor action of adrenaline or sympathetic stimulation diminish the blood flow through the skin because of diminished blood pressure or will the cutaneous outflow increase due to the opening up of possible vascular channels from the muscles? As

seen in fig. 6 stimulation of the sympathetic chains elicits an increase of the caval outflow and a marked fall of blood pressure. However, the concomitant decrease of the cutaneous outflow does not seem to be due mainly to the fall in systemic blood pressure. Acetylcholine causes, in addition to a fall in blood pressure, a significant increase in cutaneous outflow. Further, a fall of blood pressure of the same magnitude, induced by obstructing the venous inflow from the Gaddum recorder for a few seconds, did not significantly influence the blood flow from the saphenal veins. As the outflow from the saphenous veins emanates from parts of the extremities rather poor in skeletal muscles it seems justifiable to conclude that stimulation of the sympathetic trunks causes exclusively vasoconstriction. The failure of dibenamine to unmask a dilator response seems to us to make the existence of *sympathetic* vasodilators to the skin of the cat's hind legs unlikely.

BAYLISS (1901, 1902) and BACH (1946) consider that the vasodilatation in the hind limbs of the cat induced by a depressor reflex is due to increased discharge of vasodilator impulses through the dorsal roots. They deny the existence of a sympathetic vasoconstrictor tone to the hind limbs.

In our cross-circulation experiments the abdominal sympathetic chains form the only nervous connections between the upper and lower part of the recipient. The reflex vasodilatation observed in our experiments therefore must be due either to a diminished constrictor tone, or to dilator impulses or to both. In none of the experiments was a reflex vasodilatation observed in the hind limbs after dibenamine. As this substance annuls the effect of vasoconstrictor impulses and consequently abolishes the vasoconstrictor tone it seems probably that the dilator responses observed in the hind limbs were due to a reduction of a prevailing sympathetic constrictor tone.

The functional significance of the sympathetic vasodilators awaits further elucidation. Tentatively it might be assumed that they, in some way or other, are engaged in adapting the rate of blood flow to the degree of muscular activity.

Summary.

In the cat the existence of efferent sympathetic vasodilator fibres to the hind limbs has been demonstrated. This is evident from the facts

1) that vasodilatation can be produced by stimulation of the abdominal sympathetic chains by stimuli of certain characteristics,

2) that in animals given dibenamine the vasodilatation produced by sympathetic stimulation is unaffected by degeneration of afferent fibres, but reduced after degeneration of efferent fibres in the sympathetic chain.

The vasodilator fibres are confined to the muscles. No evidence was found for the existence of sympathetic vasodilators in the skin. The functional significance of the sympathetic vasodilators is obscure. Possibly they are involved in the coordination of muscular blood flow to muscular activity.

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From the Department of Physiology, University of Lund.

Cholinergic Vasodilator Nerves in the Sympathetic Outflow to the Muscles of the Hind Limbs of the Cat.

By

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In the dog cholinergic vasodilator fibres run in the sympathetic outflow. DASTRE and MORAT (1880) observed vasodilatation in the bucco-facial region on stimulation of cervical sympathetic nerves. Investigating the Rogowicz' phenomenon — the contraction of the upper lip of the dog on stimulation of the cervical sympathetics after cutting and consequent degeneration of the motor nerve to the facial muscles — EULER and GADDUM (1931) found this phenomenon probably due to the liberation of acetylcholine at vasodilator nerve endings. BÜLBRING and BURN (1935) showed that the sympathetic vasodilators to the hind limbs of the dog are cholinergic. On cats BÜLBRING and BURN only in one instance observed a vasodilatation, and that very feeble, when stimulating the sympathetic chain of an eserinizated cat. The vasodilator responses observed on cats given ergotamine, were not enhanced by eserine or abolished by atropine. BÜLBRING and BURN conclude that "in the cat the sympathetic vasodilators appear to be few and in function adrenergic. They can be demonstrated only after the injection of ergotamine". The observations of BÜLBRING and BURN were made on the isolated perfused hind limbs of dogs and cats. In order to obtain vasodilator responses to stimulation of the abdominal sympathetic chain the vascular tone of the per-

fused limbs had to be increased and maintained by a steady infusion of adrenaline. ROSENBLUETH and CANNON (1935) studied the depressor effect obtained by stimulation of the abdominal sympathetic chain on dogs and cats given ergotoxine. They observed that the depressor effects obtained in some experiments could be enhanced by eserine and more or less abolished by atropine. They concluded that in addition to adrenergic dilator fibres cholinergic vasodilators are present in the dog and cat. The discrepancy between their results and those of BÜLBRING and BURN they considered to be only quantitative, due to the use of different anaesthetics and different experimental procedures.

SHERRINGTON (1894) showed that two or three weeks after the section of the motor and sensory roots to the hind limbs of the cat, stimulation of the peroneal nerve caused contracture of the muscles. HINSEY and CUTTING (1933) found that this contracture was probably due to stimulation of sympathetic nerves, as the contracture was observed on stimulation of the grey rami of the sympathetic outflow to the hind limbs. BÜLBRING and BURN (1936) confirmed that the Sherrington phenomena can be produced in the hind legs of the dog *and the cat*.

In a previous paper FOLKOW and UVNÄS (1948 b) reported on the distribution of sympathetic vasodilators to the hind limbs of the cat. Considering the numerous reports on the existence of cholinergic vasodilators in the sympathetic outflow in the dog, and the Sherrington phenomena in the cat's hind limbs it was tempting to assume that the sympathetic vasodilators to the hind limbs of the cat were cholinergic. These experiments were undertaken to settle this issue more definitely.

Experimental.

The experimental procedure was the same as described in detail in a previous paper (FOLKOW and UVNÄS 1947 a). Eviscerated cats under chloralose-urethane were used. The blood flow from the partly isolated hind limbs was recorded according to the method described by GADDUM (1929). Intraarterial injections to the hind limbs were made into the proximal stump of the cut inferior mesenteric artery. The sympathetic chains, cut at the height of L_2 and dissected free down to the ganglion of L_5 , were stimulated electrically. To block the constrictor and reveal the dilator response to stimulation of the sympathetic chain dibenamine was given, 10–20 mg/kg slowly intravenously.

Results.

Effect of atropine. In our experiments atropine (0.5—1 mg/kg) regularly reduced the vasodilator response to stimulation of the sympathetic chain after dibenamine (fig. 1 and 2). In 6 of 12 experiments atropine completely abolished the dilator response; in the other experiments the response was reduced to at least one third of the original. Atropine did not reduce the dilator action of adrenaline or histamine.

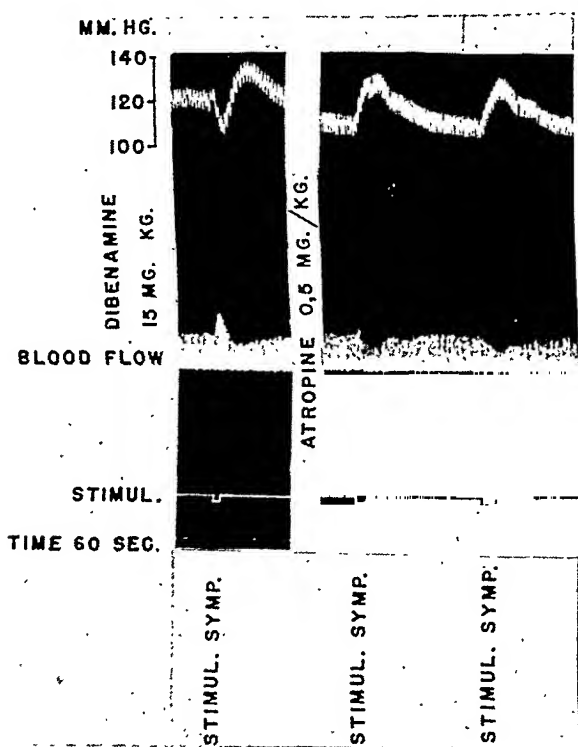


Fig. 1. Cat 3 kg. Chloralose-urethane. Dibenamine 15 mg/kg given. Effect of atropine on vasodilatation elicited by stimulation of sympathetic chains.

From above: Blood pressure, blood flow through hind limbs, stimulation marks, time marking.

Note the disappearance of the dilator response and the reappearance of a pure constrictor response.

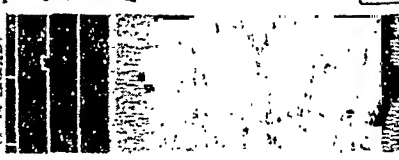
An interesting observation was made on atropinized animals. When the dilator response was abolished by atropine a vasoconstrictor response frequently reappeared (fig. 1). This constrictor effect evidently was due to the fact that dibenamine — like

A

BLOOD FLOW
STIMUL.
TIME 60 SEC.

MM.
HG.
180
160
140

STIMUL. SYMP.



DIBENAMINE 20 MG./KG.

VASOPRESSIN 1,0 I.U.

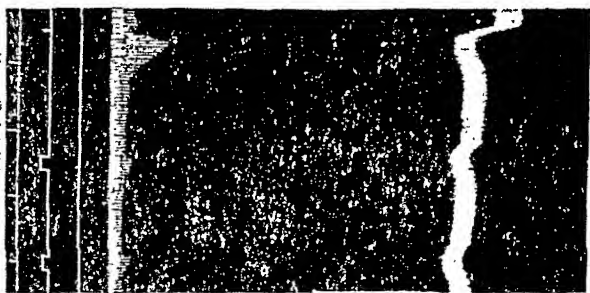
MM.
HG.
160
140
120

B

0,1 AC. CHOL. I. A.

STIMUL. SYMP.

STIMUL. SYMP.



ESERINE 0,5 MG. I.V.

C

0,1 AC. CHOL. I. A.

STIMUL. SYMP.

STIMUL. SYMP.



ATROPINE 0,5 MG./KG.

D

STIMUL. SYMP.

STIMUL. SYMP.

1 ADR. I. A.



ergotamine — does not completely block the action of the released constrictor mediator.

The effect of eserine. Our attempts to show a potentiating effect of eserine on the vasodilator responses to stimulation of the sympathetic chains met with difficulties as the combined action of dibenamine and eserine caused such a pronounced vasodilatation and depression of the blood pressure that a further vasodilatation by nerve stimulation or acetylcholine could hardly be obtained. In some experiments, where the depressor action was moderate in size or where the vascular tone was increased by adding 1—2 international units of vasopressin (0.1—0.2 ml Hypadrin, Astra) the effect of eserine could be observed. We found that in every cat — five in number — where eserine was observed to potentiate the dilator action of acetylcholine it also enhanced the dilator response to stimulation of the sympathetic chain. Fig. 2 shows the potentiating effect of eserine. In this as in other experiments the vagi were bilaterally cut in order to reduce the bradycardia caused by eserine.

The Release of Acetylcholine at Dilator Nerve Endings.

BÜLBRING and BURN (1935) observed that an acetylcholine-like substance appeared in the perfusate from the hind limbs of the dog when the abdominal sympathetic trunk was stimulated. The amount of active substance was so minute that it could only be detected by its action on the leech. Unfortunately no leeches were available in this country during the course of our experiments. We therefore resorted to the method described by DALE and FELDBERG (1934) in their study of the appearance of acetylcholine in the perfusate from the stomach of the dog on vagal stimulation.

We used cats under chloralose. The animals were eviscerated as described by FOLKOW and UVNÄS (1948 a). The sympathetic trunks

Fig. 2. Cat. 3.2 kg. Chloralose-urethane.

Potentiating effect of eserine on vasodilator response to acetylcholine, and to stimulation of sympathetic chains (10 sec, 0.5 V).

The effect of atropine.

From above: Blood pressure, blood flow through hind limbs, stimulation and injection marks, time marking.

A. Vasoconstrictor response to stimulation of symp. chain before dibenamine.

Vasodilator response to stimulation of symp. chain.

B. after dibenamine. C. after dibenamine and eserine. D. abolished by atropine.

were prepared for stimulation as previously described. The aorta and the caval vein were prepared for perfusion. Since FELDBERG and DALE (1934) have shown that the postganglionic fibres to the sweat glands of the cat's paw are cholinergic we amputated the hind legs just below the ankle joints.

The cats were given eserine 0.5—1.0 mg/kg slowly intravenously about 30 minutes prior to the perfusion to allow time for the inactivation of choline esterase. 0.5 mg atropine was given to prevent the bradycardia caused by eserine.

The hind limbs were perfused with Tyrode's solution from a pressure bottle equipped with a heater and a thermostat. The temperature of the perfusion fluid was kept at about 38° C and eserine was added to a final concentration of about 1 : 300 000—1 : 400 000.

The limbs were perfused through the aorta and the outflow collected from the caval vein. The pressure in the bottle was adjusted to give a perfusion rate of about 50 ml per minute. Care was taken to keep the rate of perfusion constant throughout the experiment. This usually did not cause any difficulties, a decrease of the outflow due to vasoconstriction occurred only to a slight degree at the beginning of a stimulation period.

After starting the perfusion, a first control sample (100 ml) was taken as soon as the outflow was uncontaminated by blood. The sympathetic trunks were then stimulated for about 2 minutes until 100 ml of perfusion fluid were collected. If a repeated stimulation was applied the perfusion was allowed to proceed uninterrupted for 5 minutes before the next control sample was taken and stimulation started again.

The pH of the samples to be tested was adjusted to about 4 by adding N/10 HCl. They were then evaporated under vacuum and the dried residue thoroughly shaken with absolute alcohol, filtered and evaporated again under vacuum. The dried residue was then dissolved in 5 ml Tyrode's solution and tested for activity on the cat's blood pressure and the rectus muscle of the frog.

Results. All of five experiments gave quite consistent results. Out of 9 samples collected during stimulation of the sympathetic trunks 7 contained a substance with the properties of acetylcholine. The active substance caused a transient fall of the cat's blood pressure. This depressor action was completely abolished by 0.1 mg atropine. The depressor action was enhanced if the cat was eserinizied. It was completely abolished if the sample was made alkaline to a pH about 9 and allowed to stand at 37° C for 30 minutes. The substance exerted the nicotine action of acetylcholine on the rectus muscle of the frog. When quantitatively compared for its muscarine and nicotine action satisfactory correspondence was observed. The activity observed corresponded to a concentration of acetylcholine of about 50 γ per litre.

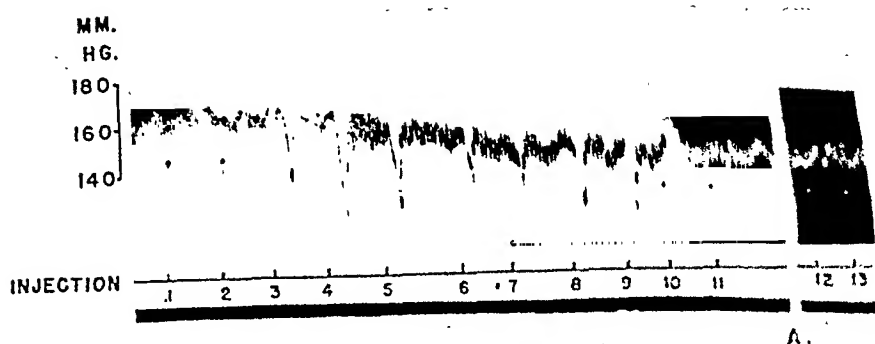


Fig. 3. Cat 2.3 kg. Chloralose.

Depressor action of perfusate from hind limbs, collected separately from caval and saphenous veins.

1. Saphenous outflow during stimulation 0.2 ml.
2. " " " " 0.8 ml.
3. Acetylcholine 0.1 %.
4. Caval outflow during stimulation 0.1 ml.
5. " " " " 0.05 ml.
6. " " " " 0.02 ml.
7. Acetylcholine 0.05 %.
8. Caval outflow during stimulation 0.05 ml.
9. Acetylcholine 0.1 %.
10. Caval outflow, control 0.1 ml.
11. " " " " 1.0 ml.
- A = atropine 0.1 mg.
12. Acetylcholine 1 %.
13. Caval outflow during stimulation 0.1 mg.

None of the 10 control samples, given in amounts ten times larger than the active samples, contained detectable amounts of acetylcholine when tested on the cat's blood pressure and the rectus muscle of the frog.

Acetylcholine is released during transmission in sympathetic ganglia (FELDBERG and GADDUM 1934). Therefore in one experiment we collected the venous outflow from the hind limbs from the femoral veins below Poupart's ligaments. In another experiment especial care was taken to ligate all branches from the aorta and the caval vein down to their passage through Poupart's ligaments. By this procedure outflow from the sympathetic ganglia could not contaminate the collected perfusate. Acetylcholine still appeared in the perfusate during stimulation of the sympathetics.

As our previous experiments (FOLKOW and Uvnäs 1948 b) indicate that the sympathetic vasodilators of the hind limbs are distributed exclusively to the muscles we in one experiment collected separately the perfusate from the caval vein and the saphenous veins, cannulated just below the knees. Fig. 3 repre-

sents the assay of samples from this experiment: 0.1 ml of the sample from the muscle collected during nerve stimulation shows a depressor action corresponding roughly to 0.1 γ acetylcholine. 1 ml of control fluid has no depressor effect. Samples from the saphenous veins, concentrated from an original volume of 25 ml to

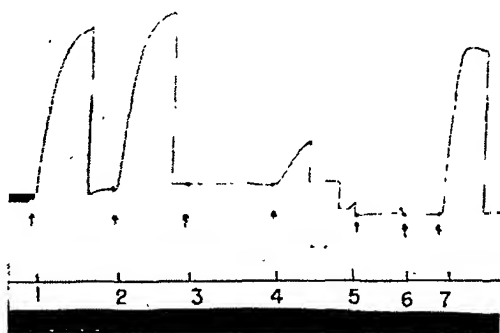


Fig. 4. Eserinizied rectus muscle of the frog. Nicotine action of perfusate from the hind limbs, collected separately from caval and saphenous veins (same samples as in fig. 3).

1. Acetylcholine 0.25 γ .
2. Caval outflow during stimulation 0.25 ml.
3. Caval outflow, control 1.0 ml.
4. Acetylcholine 0.15 ml.
5. Saphenous outflow during stimulation 1.0 ml.
6. Saphenous outflow, control 1.0 ml.
7. Acetylcholine 0.25 γ .

5 ml, had no depressor action. Fig. 4 shows the assay of the same samples on the eserinizied rectus muscle of the frog. The quantitative correspondence between the assays in the different substrates is satisfactory.

Comments.

In the dog cholinergic vasodilators are distributed in the sympathetic outflow to the bucco-facial region (EULER and GADDUM 1931) and to the muscles (BÜLBRING and BURN 1935). Adrenergic vasodilators are assumed to be confined to the intestines and the skin of the ear (BÜLBRING and BURN 1936) and the heart (among recent investigators KATZ and JOCHIM 1939, GREENE 1935, ESSEX et al. 1942).

According to current teaching the sympathetic vasodilators in the cat are adrenergic. Unable to obtain evidence for the

cholinergic nature of the sympathetic vasodilators in the hind limbs of this animal, BÜLBRING and BURN (1935) explain the discrepancy in their observations on dogs and cats by assuming that in the dog "in the course of evolution the adrenergic dilator mechanism to the muscles has been abandoned because it was necessarily inefficient. Obviously dilator fibres liberating acetylcholine are much more efficient." In a previous paper (FOLKOW and UVNÄS 1948 b) it was shown that efferent vasodilator fibres run in the sympathetic outflow to the muscles of the cat's hind legs. Our present experiments prove that these dilator fibres are cholinergic. The question then arises whether in addition to these cholinergic vasodilators there are dilator fibres of adrenergic nature in the hind limbs. This question is intimately related to the problem as to whether or not there really are any adrenergic vasodilators in the dog and cat.

The conception of the existence of adrenergic vasodilators in the dog and cat is based exclusively on indirect evidence, mainly on the observations that adrenaline in low concentrations dilates the vessels of the muscles and the heart, and that on animals given ergotamine vasodilator responses to the stimulation of sympathetic nerves is not abolished by atropine or enhanced by eserine. In our view this evidence is unsatisfactory. There is no proof that adrenaline really is the transmitter at adrenergic nerve endings in these species. On the contrary evidence has accumulated indicating that the adrenergic transmitter is a substance lacking vasodilator properties (EULER 1945, 1946, FOLKOW and UVNÄS 1948 a). The fact that atropine does not regularly annul the vasodilator response to stimulation of the sympathetics in our view is not incompatible with the opinion that the dilator fibres are cholinergic.

There are several parasympathetic effects resistant to atropine, for instance the effect of vagus impulses on the muscles of the gastrointestinal tract and of vasodilator impulses in the chorda tympani; nevertheless acetylcholine is regarded as the transmitter at the endings of these nerves. DALE and GADDUM (1930) discussing the discrepancies between the action of atropine on the effects of acetylcholine and parasympathetic impulses write: "We know nothing of the mechanism of the atropine paralysis, but for purely diagrammatic purposes we may regard it as creating a barrier, which a choline ester cannot pass. If such an ester is liberated at the parasympathetic nerve endings, to act as transmitter of the

effect of nerve impulses the latter will be paralysed completely, or partially or not at all by atropine, according as the liberation takes place wholly without, partially within or wholly within the barrier." In a later paper we will present evidence indicating that cholinergic vasodilator fibres run in the sympathetic outflow to the heart of the dog and the cat. For this reason and supported by the present results we feel justified to assume that the entire sympathetic vasodilator system in these species might be cholinergic.

EULER (1946) stresses the significant observation that the sympathomimetic agent he extracted from adrenergic nerves and various tissues innervated by such fibres, in conformity with nor-adrenaline, exhibits stronger stimulatory than inhibitory actions but that both substances still exert definite inhibitory actions on various organs. The observations of WEST (1948) agree with those of EULER in regard to the biological properties of nor-adrenaline. According to EULER "the adrenergic ergone as well as nor-adrenaline would therefore be able to elicit most of the inhibitory effect observed on sympathetic nerve stimulation, with the possible exception of those on the blood vessels where an inhibitory action is questionable". This obstacle to the assumption that nor-adrenaline or a substance with similar biological properties actually is the real adrenergic transmitter in dogs and cats would be eliminated if the sympathetic vasodilator mechanism in these species is cholinergic.

Summary.

In the cat cholinergic vasodilators in the sympathetic outflow to the muscles of the hind limbs have been demonstrated.

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Action of Adrenaline, Nor-Adrenaline and some other Sympathomimetic Drugs on the Muscular, Cutaneous and Splanchnic Vessels of the Cat.

By

BJÖRN FOLKOW, JØRGEN FROST and BÖRJE UVNÄS.

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According to current teaching the sympathetic vasodilators in the cat are adrenergic. However, recent observations indicate that the adrenergic transmitter is not adrenaline but a substance lacking vasodilator properties (EULER 1945, 1946). Experiments by FOLKOW and UVNÄS (1948) corroborate this conception. FOLKOW et al. (1948 a and b) demonstrated that cholinergic fibres run in the sympathetic outflow to the hind limbs of the cat and in the sympathetic outflow to the heart of the dog and cat. These observations make it doubtful whether vasodilators liberating adrenaline really exist in these animals. For this reason we considered it desirable to reinvestigate the vascular actions of adrenaline, nor-adrenaline and some other sympathomimetic drugs.

Experimental.

Experiments were performed on cats under chloralose-urethane anaesthesia. The experimental procedure for observations of muscular and cutaneous vessels was the same as described by FOLKOW and UVNÄS (1948). In principle: After evisceration the animal was partly divided into an upper and a lower part by mass ligation of the abdominal wall and the longitudinal muscles of the back at the height of the fifth lumbar vertebra. The blood flow in the hind limbs

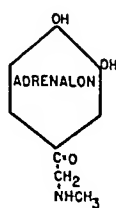
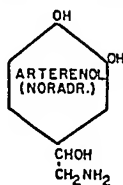
was recorded by the technique described by GADDUM (1929). The drugs subjected to investigation were injected intraarterially into the proximal stump of the severed inferior mesenteric artery.

Observations on the splanchnic vessels were made in the following way. The abdominal cavity was opened by a midline incision. The spleen and the great omentum were removed. The pyloric region and the colon just distally to the inferior mesenteric artery were ligated. Heparin 5 mg per kg was given. The portal vein and the main trunk of the superior mesenteric artery were dissected free. The artery was temporarily clamped, the portal vein ligated and two cannulas inserted, one proximally, the other distally to the ligature. The superior mesenteric artery was then severed a few mm distally to the point where it branches off from the aorta and a short rubber tubing attached between the two cut ends of the artery. The rubber tubing was used for intraarterial injections into the splanchnic vascular bed. The clamp was then removed from the superior mesenteric artery and the portal outflow directed to a Gaddum recorder. The outflow from the recorder was returned to the proximal part of the ligated portal vein. In some experiments the left splanchnic nerve was prepared free, cut just below the diaphragm and placed in a Ludwig electrode. The vessels of the left adrenal were ligated. The abdominal incision was then closed, care being taken not to interfere with the blood flow to and from the recorder. The blood pressure was recorded in the common carotid artery.

Results.

Action on muscular vessels. The constitution of the sympathomimetic drugs investigated is shown in fig. 1. Their action on the blood flow in the hind limbs was recorded in experiments on 14 cats. Nor-adrenaline proved to be the most potent vasoconstrictor drug, the minimal active dose being 0.1–1.0 γ . As adrenaline in low concentrations is known to exert a vasodilator action on muscular vessels some of the experiments were performed on skinned hind limbs. Under these conditions nor-adrenaline still elicited pure vasoconstrictor responses. An experiment of this type is shown in fig. 2. Adrenaline in the doses 0.1, 1.0 and 2.0 γ elicited a vasodilator response, higher doses, 3.0 and 5.0 γ , caused predominant vasoconstriction. Nor-adrenaline in the doses 0.1, 1.0 and 2.0 γ induced pure vasoconstriction. Under the influence of dibenamine, 20 mg per kg, the vasoconstriction induced by adrenaline was reversed to a pure vasodilation (fig. 3). On the other hand dibenamine partially or completely blocked but did not reverse the constrictor action of nor-adrenaline (fig. 3). In a minority of experiments with doses of nor-adrenaline of 10 γ or

DIHYDROXY BENZENE:



MONOHYDROXY

BENZENE:

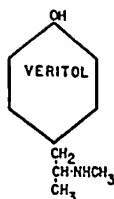
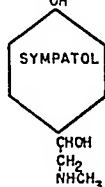
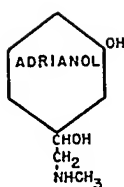


Fig. 1.

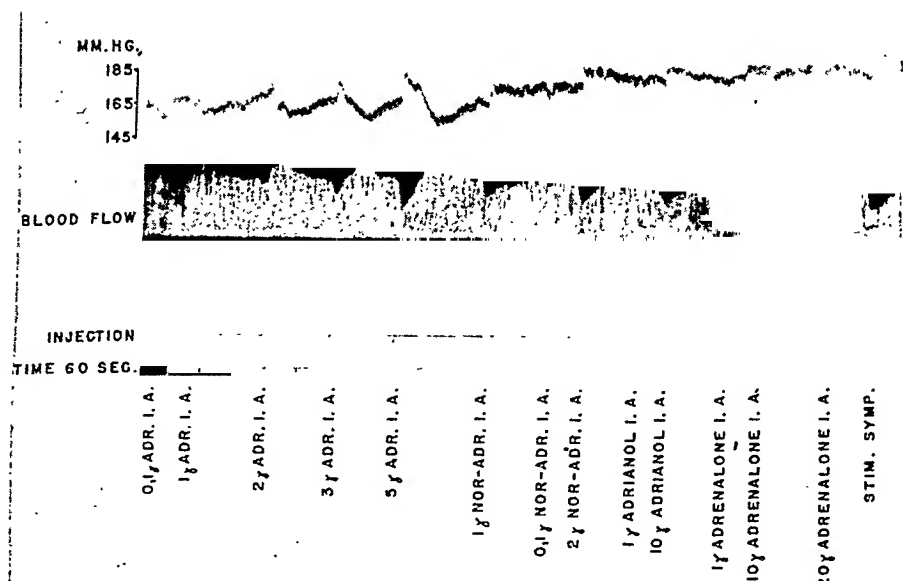


Fig. 2. Cat 3.8 kg. Chloralose-urethane. Limbs skinned.

Vasomotor responses to intraarterial injections of adrenaline, nor-adrenaline, adrenalone and adrianol.

From above: Blood pressure, blood flow through hind limbs, injection marks, time marking.

more a very slight vasodilator action on the muscular vessels was revealed under the influence of dibenamine.

Adrenalone (adrenone, stryphnone), adrianol (neo-synephrine) and suprifen exerted a considerably weaker vasoconstriction than adrenaline and nor-adrenaline; of these drugs 10—20 γ had to be given to elicit a significant vasoconstriction. Dibenamine com-

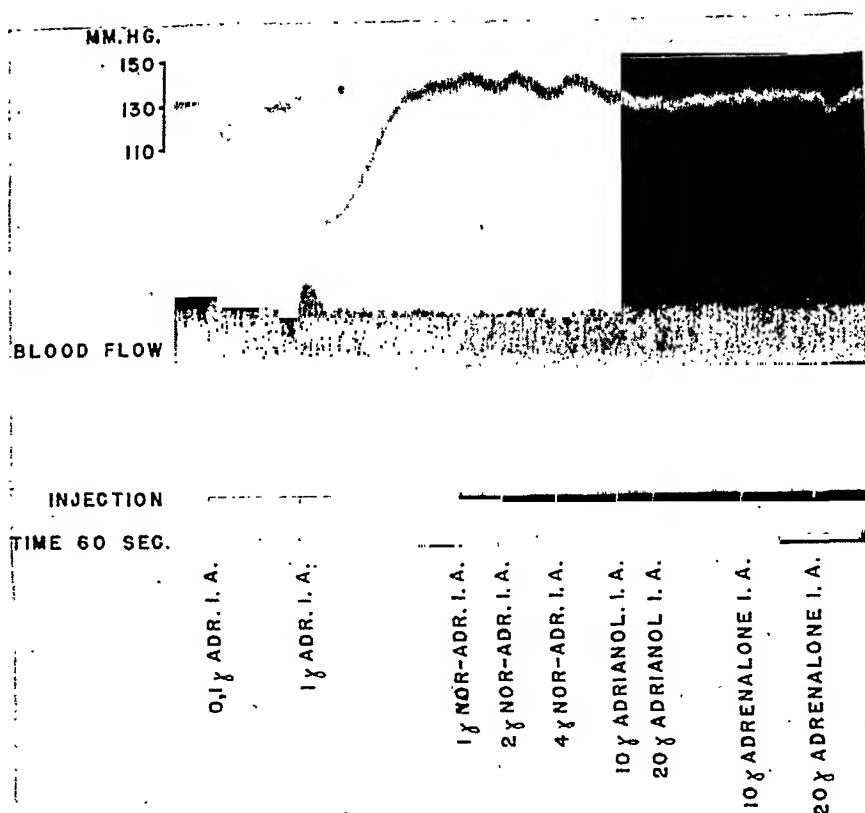


Fig. 3. Cat 3.8 kg. Chloralose-urethane. Limbs skinned.

Dibenamine 20 mg/kg given.

Vasomotor responses to intraarterial injections of adrenaline, nor-adrenaline, adrenalone and adrianol.

From above: Blood pressure, blood flow through hind limbs, injection marks, time marking.

pletely annulled these constrictor effects. As seen in fig. 3 a slight vasodilatation appeared after 20 γ adrenalone. It was regularly observed that when the doses of adrenalone, adrianol and suprifren were increased to 100 γ or more a slight vasodilatation occurred under the influence of dibenamine. Sympatol and veritol were even less potent vasoconstrictors, about 100 γ being the minimal effective dose. The constrictor action of these two drugs was completely abolished by dibenamine. As with the other substances a slight vasodilator action appeared under dibenamine when the doses of sympatol and veritol were increased 10—100 times the minimal active dose.

Action on cutaneous vessels. In five experiments the blood flow was separately collected from the caval and the saphenous veins cannulated 2—3 cm below the knees. No vasodilator action of

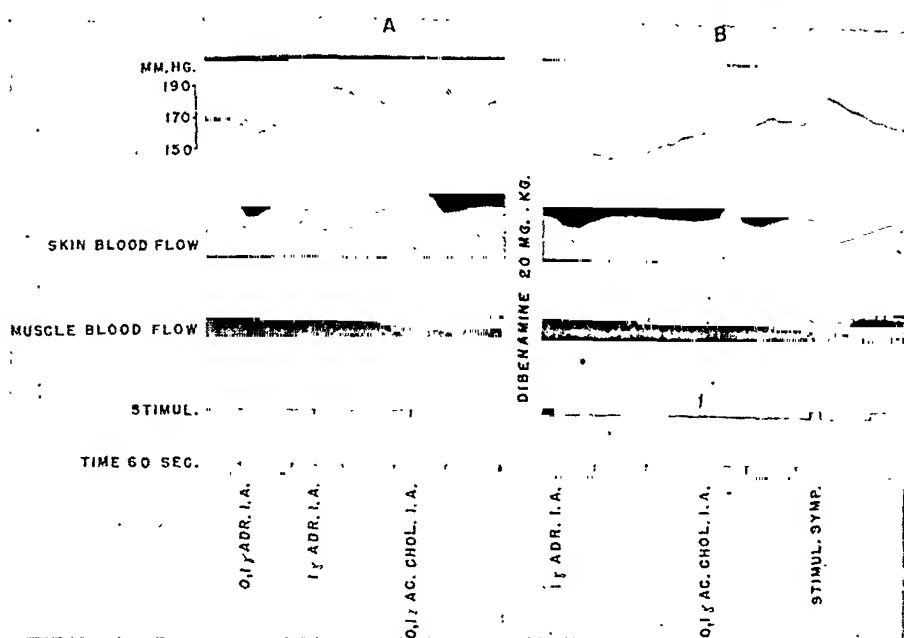


Fig. 4. Cat 2.8 kg. Chloralose-urethane.

Vasomotor responses in skin and muscles to adrenaline and acetylcholine. From above: Blood pressure, outflow from saphenous veins, outflow from caval vein, injection marks, time marking.

A) before dibenamine, B) after dibenamine 20 mg/kg.

adrenaline was observed on the vessels of the skin. As seen in fig. 4 0.1 γ adrenaline increases the caval outflow but decreases the outflow from the saphenous veins. 0.1 γ acetylcholine increases caval as well as cutaneous flow concomitantly with a pronounced fall in blood pressure. Evidently acetylcholine dilates both the muscular and cutaneous vessels. A blood pressure fall of the same magnitude as caused by acetylcholine produced by obstructing the venous return from the GADDUM recorder, did not significantly reduce the flow in skin. The decrease in the saphenous outflow observed after vasodepressor doses of adrenaline thus indicates a vasoconstrictor action of adrenaline in the skin.

Dibenamine in doses that reversed the vasoconstrictor response of the muscular vessels to adrenaline to a vasodilatation did not reverse the constriction in the skin. The vasoconstriction in the skin was largely, but usually not completely blocked by dibenamine (see fig. 4).

Action on splanchnic vessels. In experiments on 6 cats the action of adrenaline and nor-adrenaline on the splanchnic vessels was recorded. Both substances elicited exclusively vasoconstrictor re-

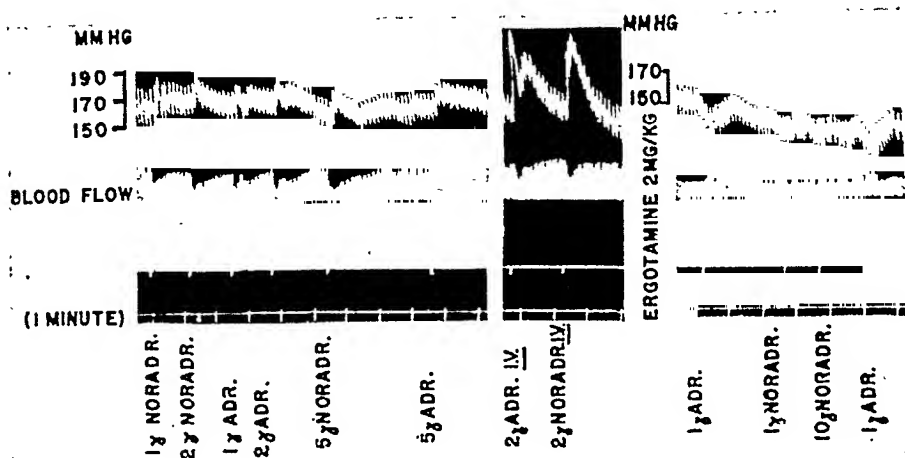


Fig. 5. Cat 3.2 kg. Chloralose-urethane.

Vasomotor responses to adrenaline and nor-adrenaline given intraarterially and intravenously. Influence of ergotamine 2 mg/kg.

From above: Blood pressure, splanchnic blood flow, injection marks, time marking.

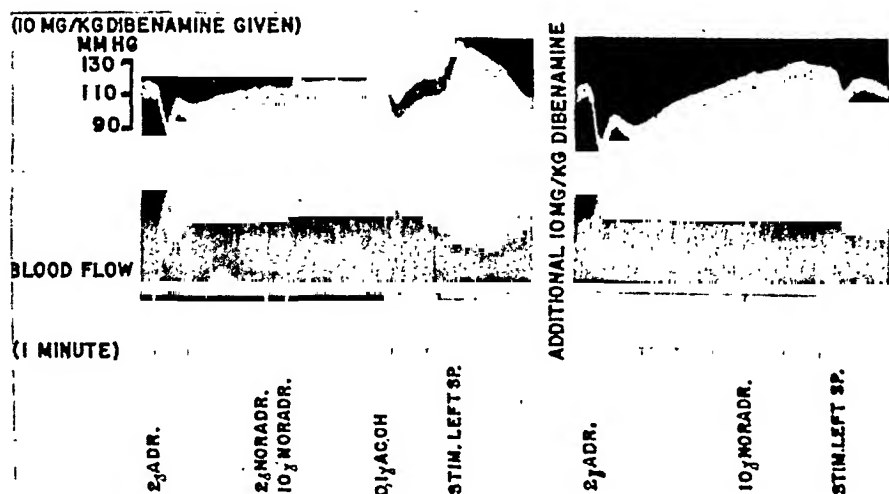


Fig. 6. Cat 3.4 kg. Chloralose-urethane. Both adrenals removed.

Vasomotor responses to adrenaline, nor-adrenaline and stimulation of left splanchnic nerve under the influence of dibenamine.

From above: Blood pressure, splanchnic blood flow, injection and stimulation marks, time marking.

sponses of approximately equal magnitude (fig. 5). Also when given by the intravenous route in minimal effective doses both substances showed constrictor action (fig. 5). Ergotamine (in fig. 5 two mg per kg) reversed the response to adrenaline from vasoconstriction to a slight vasodilatation. The action of nor-adren-

aline was simply annulled. In fig. 6 is shown the course of events under the influence of dibenamine. The vasoconstriction produced by adrenaline changes into a vasodilatation, the vasoconstriction produced by nor-adrenaline is simply annulled. The figure also demonstrates the action of dibenamine on the vasomotor response to stimulation of the left splanchnic nerve. The original vasoconstrictor response disappears and a small vasodilator effect is revealed.

Comments.

Our experiments demonstrate that nor-adrenaline is the most potent vasoconstrictor drug so far investigated. On intraarterial injection, in doses that reasonably might be considered as within physiological limits, it constricts the muscular, cutaneous and splanchnic vessels of the cat. Under physiological conditions the substance entirely lacks vasodilator action. Our observations are in harmony with the conception recently presented by EULER (1945, 1946) that nor-adrenaline or a substance with its biological properties might be the adrenergic transmitter, at least in the cat.

FOLKOW et al. (1948 a and b) demonstrated that cholinergic fibres run in the sympathetic outflow to the muscles of the cat's hind limbs and to the heart of the cat and dog. These observations were interpreted as indicating that the sympathetic vasodilators to the muscles of the cat are cholinergic and the sympathetic vasodilators to the heart might be cholinergic. No sympathetic vasodilators have been shown to exist in the skin of the cat (FOLKOW and UVNÄS 1948). Stimulation of the splanchnic nerves on cats given ergotamine or dibenamine elicits a slight vasodilatation. This dilatation is not abolished by atropine. Similar findings on dogs led BÜLBRING and BURN (1936) to assume that the vasodilators running in the splanchnic nerves were adrenergic. HARTMAN (1915) and CLARK (1944) claimed that the action of adrenaline on the splanchnic vessels of the cat is exclusively constrictor. This is in agreement with our observations. In our experiments adrenaline injected from the artery constricts, never dilates the splanchnic vessels. This fact makes it unlikely that adrenaline is the transmitter at vasodilator nerve endings in the splanchnic region. From the present and previous observations (FOLKOW, HÆGER and UVNÄS) we consider it unlikely that sympathetic vasodilators liberating adrenaline really exist in the cat.

The vasodilatation observed on stimulation of the splanchnic nerves of the dog and cat given ergotamine is by most investigators interpreted as due to the activation of sympathetic vasodilator fibres (DALE 1913, BÜLBRING and BURN 1935 and others). Other investigators, for instance GERNANDT and ZOTTERMAN (1946) are inclined to refer the vasodilatation to the activation of afferent fibres. In our view the very minute vasodilatation observed on stimulation of the splanchnic nerves might be referred simply to haemodynamic factors. Dibenamine and ergotamine block the action of vasoconstrictor impulses to the splanchnic region but do not interfere with the inhibitory action of splanchnic impulses on smooth muscles. The small transient vasodepressor effect observed on splanchnic stimulation could possibly be due to a decrease of the peripheral vascular resistance occurring when the smooth muscles of the gastro-intestinal tract relax.

If adrenaline, as we argue, is not the transmitter at adrenergic vasomotor nerve endings, what is the function of adrenaline released from the adrenals? Since adrenaline in physiological concentrations dilates the vessels in skeletal muscles and reduces the blood flow in the splanchnic and cutaneous regions, this agent is admirably qualified to accentuate and enhance the redistribution of the blood flow from relatively inactive to active regions as originally suggested by CANNON.

Summary.

Adrenaline in low concentrations dilates, in high concentrations constricts the muscular vessels of the cat.

Nor-adrenaline, adreneone (adrenone, stryphnone), adrianol (neo-synephrine), suprifren, sympatol and veritol exert exclusively constrictor actions on muscular and cutaneous vessels. Nor-adrenaline is the most potent vasoconstrictor substance, the minimal active dose being 0.1—1 γ for a cat.

On the cutaneous and splanchnic vessels the action of adrenaline is exclusively vasoconstrictor.

Dibenamine and ergotamine reverse the constrictor action of adrenaline to a dilator action on the muscular and splanchnic vessels. Under the influence of these drugs the constrictor action on cutaneous vessels is more or less annulled but not reversed. The constrictor action of nor-adrenaline on muscular, cutaneous and splanchnic vessels is blocked but not reversed by these drugs.

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Cholinergic Fibres in the Sympathetic Outflow to the Heart in the Dog and Cat.

By

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The coronary vessels are richly supplied with parasympathetic and sympathetic nerves. According to current teaching constrictor fibres run in the vagi, dilator fibres in the sympathetics, mainly via the stellate ganglia (MAAS 1899, WIGGERS 1909, MORAWITZ and ZAHN 1914, ANREP and SEGALL 1926, ESSEX et al. 1942, GREGG and SHIPLEY 1944). However, several investigators claim that constrictor as well as dilator fibres occur both in the vagi and the sympathetics, because stimulation of these nerves in some experiments elicits constriction, in others dilatation. GREENE (1935) refers the opposed results obtained on nerve stimulation to the anatomical distribution of the sympathetic coronary nerves. According to GREENE the sympathetic outflow contains constrictor as well as dilator fibres. Some of the sympathetic fibres run upward in the sympathetic chain in the neck, cross to the nodose ganglion and course downward in the vagi to the heart. Experimenting on the fibrillating heart of the dog KATZ and JOCHIM (1939) obtained results divergent from all others. They claim that the vagal coronary nerves are exclusively dilators, cholinergic in character. They consider the sympathetic coronary nerves to be constrictors as well as dilators, the dilator fibres being adrenergic.

Among recent investigators ECKENHOFF et al. (1947) and FOLKOW et al. (1948) have shown that acetylcholine given intra-

arterially in minute doses (0.01—10 γ) dilates the coronary arteries. Observations by EULER (1945, 1946) and FOLKOW and UVNÄS (1948) indicate that the adrenergic transmitter lacks vasodilator properties. If this is so, the coronary dilators are not likely to be adrenergic. In our view it is more reasonable to assume that they are cholinergic. To support this assumption we attempted to obtain evidence to show that acetylcholine is liberated on stimulating the sympathetic coronary dilator fibres.

Experimental.

The experiments were performed on five cats and five dogs. The animals were anaesthetized with chloralose, the trachea cannulated and both the vago-sympathetic trunks severed in the neck. In order to inhibit the enzymatic destruction of acetylcholine, eserine, 1 mg per kg, was injected slowly intravenously. To prevent the bradycardia and mucous secretion elicited by eserine 0.5 mg atropine was given. 30 minutes later the animal was prepared for perfusion of the coronaries. The heart was exposed by removing the anterior part of the thoracic wall. Both stellate ganglia were prepared free and all branches severed except those directed to the heart. The aorta just distally to the aortic arch, the brachiocephalic artery, the left subclavian artery, the superior and inferior caval veins and the vena azygos were ligated. The pericardium was opened, the pulmonary artery ligated and cannulated proximally to the aortic arch. Finally the hili of the lungs were ligated and the perfusion was started. The coronaries were perfused from a pressure bottle via the aorta and the perfusate collected from the pulmonary artery. The perfusion fluid was oxygenized, kept at a temperature of 37° C and contained eserine 1 : 400 000. The perfusion pressure was adjusted to about 100 mm Hg. Under these conditions the perfusate amounted to about 75 ml per minute in a cat, or a small dog — weighing less than 5 kg. — When after a few minutes the perfusion fluid became colourless, *i. e.* practically free from blood, a first control sample of 200 ml was taken. The stellate ganglia were then stimulated. Sherrington electrodes and a Thyatron stimulator were employed. 200 ml perfusion fluid was collected and then stimulation discontinued. Five minutes later a second control sample was taken and then the ganglia stimulated again. Throughout the experiment care was taken to keep the perfusion at a constant rate, which was quite easily done since stimulation of the stellate ganglia only initially caused a slight increase of the coronary outflow.

The collected samples were assayed for their acetylcholine activity. As no leeches were available in this country we resorted to the procedure described by FELDBERG and DALE (1934). The samples were acidified to pH about 4 by adding N/10 HCl and then evaporated to dryness in vacuum. After extraction of the residue with absolute

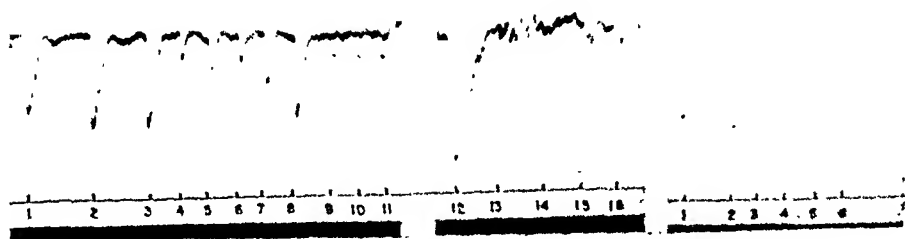


Fig. 1 a.

Fig. 1 b.

Fig. 1 a. Cat. 2.8 kg. Chloralose. Depressor action of coronary perfusates from a cat.

1. Acetylcholine (A.ch.) 0.1 %. 2. Stimulation 0.1 ml. 3. Stimulation 0.05 ml. 4. Stimulation 0.01 ml. 5. Stimulation 0.02 ml. 6. A.ch. 0.02 %. 7. A.ch. 0.05 %. 8. A.ch. 0.1 %. 9. Control before stimulation 0.1 ml. 10. Control before stimulation 1.0 ml. 11. Control after stimulation 0.1 ml. 12. Stimulation 0.1 ml. (Note the marked potentiation of the depressor effect at 12. The cat showed symptoms of eserine intoxication (see text). 13. Atropine 0.1 mg. 14. Stimulation 0.1 ml. 15. A.ch. 1 %. 16. A.ch. 1 %.

Fig. 1 b. Eserinized rectus muscle of frog (eserine 1:100 000). Nicotine action of coronary perfusate from cat (the same samples as in fig. 1 a).

1. Stimulation 0.7 ml. 2. Control before stimulation 0.7 ml. 3. Control after stimulation 0.7 ml. 4. A.ch. 1 %. 5. Stimulation 0.7 ml. (incubated at 37° C and pH 8.5 for 30 minutes). 6. A.ch. 1 %.

alcohol and a repeated evaporation in vacuum the dried material was dissolved in 5 ml Tyrode's solution. The perfusion fluid was in this way concentrated 40 times.

The samples were tested for their muscarine action on the cat's blood pressure and for their nicotine action on the eserinized rectus muscle of the frog. Before testing on the frog's rectus the fluids were diluted with water 1.4 times their volume.

Results.

In all experiments a substance with the biological properties of acetylcholine appeared in the coronary perfusate during stimulation of the stellate ganglia. On intravenous injection this substance elicited a fall of the cat's blood pressure. This depressor action was enhanced by eserine and abolished by small amounts of atropine. As seen in fig. 1 a and 2 a the active agent showed the same concentration-action curve as acetylcholine. It elicited a contraction of the eserinized rectus muscle of the frog. The samples lost their activity if alkalized to pH 8—9 and allowed to stand at 37° C for 30 minutes. When a sample was tested for its muscarine and nicotine action a good quantitative correspondence was obtained between the two tests. The

concentrations of acetylcholine corresponding to the activity of the different samples appears in the table. As seen, out of 20 samples collected during stimulation only one lacked activity, while all the control samples were inactive, even if given in doses 10 times higher than samples obtained during stimulation (see fig. 1 a and 2 a).

Since eserine is not destroyed during the extraction processes it appears in high concentrations in all samples. Maximally, *i. e.* if no destruction occurs the concentration of eserine amounts to 1 : 10.000. Especially when large volumes of the control samples are given the total amount of eserine injected into a cat during an assay will be sufficient to potentiate the action of acetylcholine.

Figs. 1 a and 1 b show assays on the muscarine and nicotine action of a coronary perfusate from a cat. The figures illustrate all the properties of the active agent described above.

Discussion.

The fact that in the dog and cat the vagi carry constrictor and the sympathetics from the stellate ganglia dilator fibres to the coronaries, has by many investigators been taken as indicating that the constrictor nerves are cholinergic and the dilator nerves adrenergic. If this conception is correct one would expect acetylcholine to constrict and the adrenergic transmitter to dilate the coronaries. However, acetylcholine dilates the coronaries of the mammalian heart. It is true that adrenaline is generally considered to be a potent coronary dilator, but recent observations indicate that this substance is not the transmitter at adrenergic nerve endings in the dog and cat. The adrenergic transmitter probably is a substance with exclusively vasoconstrictor action (EULER 1945, 1946, FOLKOW and UVNÄS 1948). In our view present information about the biological properties of the transmitter substances favour the idea that the coronary dilators are cholinergic and the constrictors adrenergic rather than the reverse. GREENE (1935) brought anatomical evidence to support the idea that the constrictor fibres in the dog are adrenergic, when he showed that efferent sympathetic constrictor fibres to the coronaries run in the vagi of this animal. We have found that a substance with the biological properties of acetylcholine appears in the perfusate from the coronaries of the dog and cat during stimulation of the stellate ganglion. Since sympathetic vasodilator

Acetylcholine Activity of Coronary Perfusates from Dog and Cat.

Animal	Sample	Concentration of acetylcholine corresponding to the activity of the perfusates (γ /lit.).
Cat no. 1 (fig. 1 a)	C1	0
	S1	25 γ
	C2	0
	S2	25 γ
Cat no. 2	C1	0
	S1	18 γ
	C2	0
	S2	12 γ
Cat no. 3	C1	0
	S1	18 γ
	C2	0
	S2	38 γ
Cat no. 4	C1	0
	S1	25 γ
	C2	0
	S2	0
Cat no. 5	C1	0
	S1	25 γ
	C2	0
	S2	25 γ
Dog no. 1 (fig. 2 a)	C1	0
	S1	25 γ
	C2	0
	S2	25 γ
Dog no. 2	C1	0
	S1	25 γ
	C2	0
	S2	3 γ
Dog no. 3	C1	0
	S1	25 γ
	C2	0
	S2	3 γ
Dog no. 4	C1	0
	S1	18 γ
	C2	0
	S2	30 γ
Dog no. 5	C1	0
	S1	38 γ
	C2	0
	S2	30 γ

C = Control.

S = Perfusates collected during stimulation of the sympathetics.

fibres, cholinergic in character, are widely distributed in the dog and cat (BÜLBRING and BURN 1935, FOLKOW et al. 1948) we consider it reasonable to assume that the appearance of acetylcholine in the coronary perfusate is due to the activation of cholinergic dilator fibres in the sympathetic outflow to the heart.

Summary.

A substance with the biological properties of acetylcholine appears in the coronary perfusate of the dog and cat during stimulation of the stellate ganglia.

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The effect of Atropine, Acetylcholine, Eserine and Di-Isopropylfluorophosphate on the Gastric Secretion of the Cat.

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It is a well known fact that vagal impulses stimulate the gastric secretion. The HCl, the peptic as well as the mucus glands are activated. Recent investigations on this subject have been reported by VINEBERG (1931), BOWIE and VINEBERG (1935), TOBY (1936), UVNÄS (1942), BJÖRKMAN, NORDÉN and UVNÄS (1943), KOMAROW (1947), STAVRAKY (1948). DALE and FELDBERG (1934) found an increased amount of acetylcholine in the eserinizied blood from the stomach of dogs during vagal stimulation. This increase was evidently due to the transmission of vagal impulses to the muscles of the stomach. Whether some part of the acetylcholine emanated from the activation of the secretory cells is obscure. However, conforming to the prevalent theory of acetylcholine as the parasympathetic transmitter it is per analogiam commonly assumed that this substance is involved in the activation of the gastric secretory mechanism by vagal impulses. A definite proof of this assumption is still lacking, all evidence being indirect, chiefly based on the fact that parasympathicomimetic drugs have been observed to evoke gastric secretion.

The effect of acetylcholine on the gastric secretion of man and dog has been studied by numerous investigators. The results vary. FAREY and DERON (1931), WILKINSON (1932), MERKLEN, WARTER and KABABER (1932) and others investigated the effect of acetylcholine on the gastric secretion of man. When 100—200 mg

of the drug was given subcutaneously an occasional increase of the acidity of the gastric juice was found. Quite negative results were reported by DA COSTA (1935). GEBHARDT and KLEIN (1933) observed an increase of the acidity only in a few cases. More often an augmentation of the "wässerige" secretion and always an increased output of chlorides was seen. On Heidenhain pouch dogs NECHELES and co-workers (1938) observed an augmentation of volume, acidity and peptic activity of the gastric juice after repeated subcutaneous doses of 10—100 mg acetylcholine every 10 minutes. Prostigmine was considered to enhance the secretory effect of acetylcholine. 50 γ of this drug was found to increase the gastric secretion. GRAY and IVY (1937) claimed that the combined administration of acetylcholine and eserine evoked a gastric secretion in total pouch dogs. STAVRAKY (1943) reports similar results on Heidenhain pouch dogs.

VINEBERG (1936)¹ injected acetylcholine directly into the stomach arteries of dogs (through the splenic artery) and obtained a thick mucous fluid without any free acid but with a peptic activity that varied with the dose of acetylcholine given. Large doses gave less pepsin than small ones. STAVRAKY¹ (1942) found under similar conditions that large doses of acetylcholine administered intraarterially evoked the secretion of a thick mucous juice, devoid of both free acid and pepsin. He did not believe that the absence of pepsin was due to its rapid inactivation in the slightly acid or alkaline juice. When histamine was given intraarterially, a juice highly acid but poor in pepsin was obtained. If histamine and acetylcholine were given concomitantly the juice secreted was highly acid and very rich in pepsin. It seemed as if acetylcholine was able to stimulate the peptic cells only in cooperation with histamine (BABKIN 1944).

NECHELES and co-workers observed in their experiments that histamine and acetylcholine act as synergists. A slow secretion evoked by one of these drugs was considerably enhanced by the concomitant administration of the other.

The effect of mecholyl (acetyl methyl choline) has been reported in many papers. MYERSON and co-workers (1936) observed a decrease, ABBOT (1933) occasionally an increase of the gastric acidity in man after subcutaneous injection of the drug. FERGUSON and SMITH (1935) found an inhibition of the gastric secretion of

¹ The unpublished results of these investigators quoted by BABKIN (1944) were not known to us when our experiments were started.

monkeys after rather heavy doses of mecholyl. GRAY and IVY (1937) reported that on total pouch dogs small doses stimulated but large doses inhibited the HCl secretion. On Heidenhain pouch dogs NECHELES and co-workers observed mecholyl to be a strong stimulant in all doses. STAVRAKY (1943) agrees with GRAY and IVY that mecholyl in small doses stimulates but in large doses inhibits the gastric secretion. The gastric juice obtained by STAVRAKY was of high peptic activity. Most investigators cited above are of the opinion that mecholyl stimulates the mucous cells. From the results on dogs it can be concluded that mecholyl given in appropriate doses stimulates all the main secretory cells of the stomach.

Another stable choline ester subjected to investigation is carbaminoylcholine chloride (doryl, lentin). NÖLL (1932) reported a copious gastric secretion on fistula dogs after the subcutaneous injection of this drug. GOODMAN (1938) considers it to be the most potent secretory agent among the parasympathetic drugs. The secretion observed by him on dogs with isolated pouches was highly acid and rich in pepsin.

Pilocarpine is a potent gastric secretory stimulant on dogs. Both the HCl, pepsin and mucus cells are activated (TOBY 1936, VINEBERG and BABKIN 1931). The gastric cells of the cat seem to differ from those of the dog in regard to the response to pilocarpine. None or only a very scanty secretion could be evoked (BOLTON and GOODHARDT 1931).

The reports on the effect of parasympathetic drugs on the gastric secretion of the cat are surprisingly scarce. We performed some experiments to investigate the effect of atropine, acetylcholine, eserine and di-isopropyl-fluorophosphate on the gastric secretion of that animal.

Experimental.

Cats under chloralose-urethane anaesthesia were used (50 mg chloralose and 500 mg urethane per kg body weight). The trachea was cannulated and to avoid cerebral influences on the gastric cells, in most experiments the vagi were cut in the neck. To obtain pure gastric juice the oesophagus and the duodenum just distal to the pylorus were ligated. The gastric juice was collected in 15-minute periods through a cannula inserted into the stomach wall (Uvnäs 1945). The animals were left to recover for one hour before the beginning of an experiment.

An alternating electric current of about 40 cycles per second was used for stimulation of the vagi. The vagi were alternately stimulated for short periods, using a metronome with about one stroke per second in the circuit.

The acidity was determined by colorimetric titration, N/10 NaOH being used as the base. The peptic activity of the juice was determined by the modification of the Anson and Mirsky method described by UVNÄS (1945).

Results.

Atropine. As expected atropine inhibited the gastric secretion induced by vagal stimulation. It was regularly seen that 0.5—1.0 mg per kg body weight almost stopped the secretion induced by vagal stimulation. A small secretion remained that could not be entirely abolished until doses that were highly toxic were used.

Acetylcholine. The negative results reported after subcutaneous administration of acetylcholine are not surprising. Due to the rapid enzymatic destruction of the drug in the tissues and the blood, most if not all of it must be destroyed before it reaches the gastric cells. In order to diminish the uncertainty of the dosage we gave acetylcholine by the intravenous route. Amounts from 0.01 to 1,000 γ per kg body weight and minute were given. No stimulation of the HCl glands was observed. On the contrary the initial basal acid secretion was depressed and the reaction to Congo of the scanty secretion turned from positive to negative, indicating the disappearance of the free HCl. With the heavy dose of 1,000 γ a slight increase of the mucous secretion occurred. As the absence of any stimulating effect on the HCl secretion might be due to the rapid destruction of acetylcholine on its way to the secreting cells the drug was in several experiments given by the intraarterial route. Continuous infusions were made into the distally ligated splenic artery. The results were still negative. Doses from 10—200 γ per kg body weight and minute changed the reaction of the juice to Congo negative. With large doses the secretion of a thick mucous juice was observed. The absence of HCl secretion was not due to any inability of the parietal glands to secrete. As seen from fig. 1 after two hours of intraarterial injection of acetylcholine the HCl cells responded to vagal stimulation with a copious secretion of HCl.

Eserine. As the inability of acetylcholine to stimulate the HCl cells could still be due to an enzymatic destruction of the

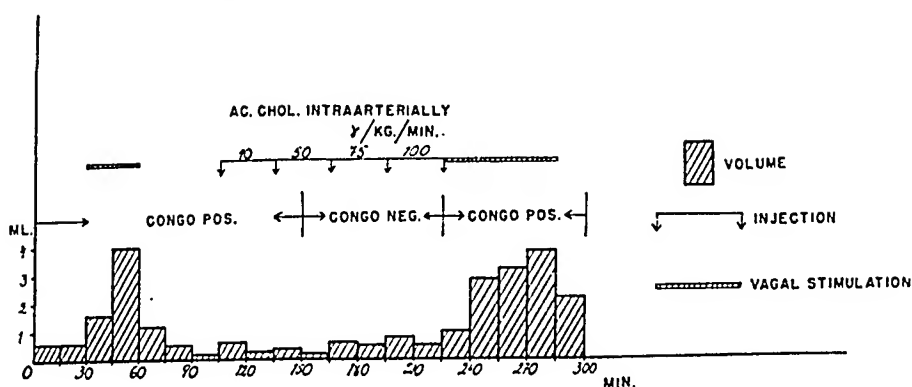


Fig. 1. Cat 3 kg. Gastric secretion during vagal stimulation before and after continuous intraarterial injection of acetylcholine in doses from 10–100 γ per kg body weight and minute.

substance, experiments were performed on eserized animals. It was then observed that eserine itself, if given intramuscularly or subcutaneously in adequate doses, evoked a copious gastric secretion. About 1 mg per kg body weight was the threshold dose. The juice obtained was of high acidity and rich in pepsin. An experiment is shown in fig. 2 where 5 mg eserine was given subcutaneously. The secretion usually started in 15–30 minutes, reached a peak about an hour later and then gradually declined in 1–2–3 hours. The free acidity during the peak of the secretion was usually over 150 m. eq. per litre and the peptic activity was

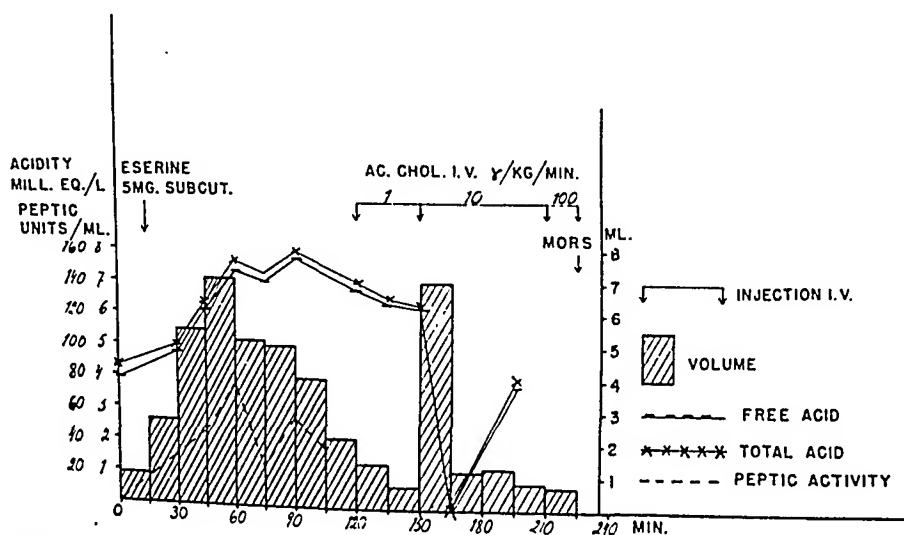


Fig. 2. Cat 3.2 kg. Gastric secretion after eserine 5 mg subcutaneously, and the continuous intravenous injection of acetylcholine in doses from 1 to 100 γ per kg body weight and minute.

considerable. The effect of eserine on the peptic secretion was further studied in experiments where the secretion of a pepsin free juice was obtained by continuous intravenous injection of histamine (BJÖRKMAN, NORDÉN and UVNÄS). A pronounced output of pepsin occurred. The peptic secretion began to rise about

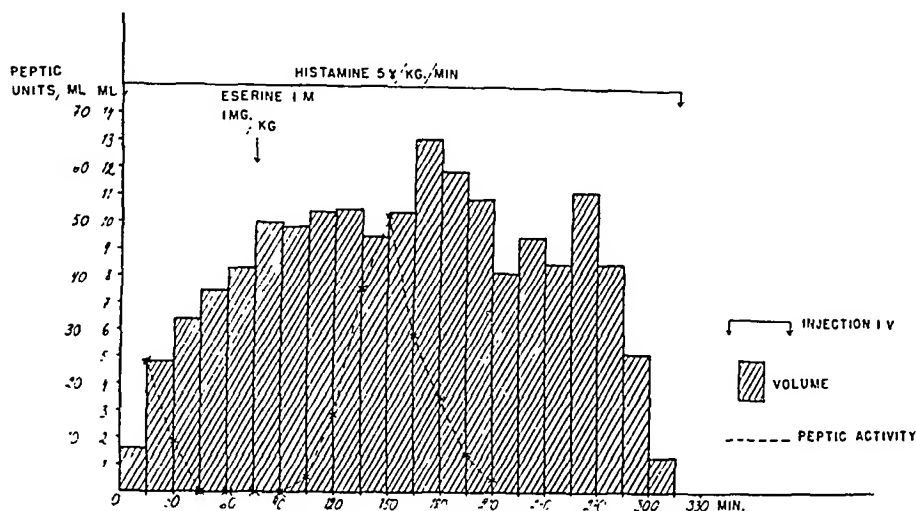


Fig. 3. Cat 2.8 kg. The effect of eserine on the peptic secretion. Gastric secretion is initiated and maintained by continuous intravenous injection of histamine. The arrow indicates eserine 1 mg intramuscularly per kg body weight.

30 minutes after the administration of eserine, reached a peak 30—60 minutes later, and then gradually declined. The effect of 1 mg eserine per kg body weight is seen in fig. 3.

The secretory effect of eserine remained after bilateral vagotomy and splanchnicotomy. It was completely abolished by 1 mg atropine per kg body weight intravenously or intramuscularly.

Acetylcholine and eserine. Eserine was given in subthreshold doses, followed by acetylcholine. Neither by the intravenous nor by the intraarterial route could any secretion of an acid juice be evoked. The reaction of the juice always turned negative to Congo, and the secretion of a thick mucous juice appeared as the doses of acetylcholine were increased. In numerous experiments doses from 0.001 γ per kg body weight up to doses that caused respiratory disturbances were given. In some experiments acetylcholine was injected after the secretion induced by eserine had passed its peak. No significant increase of the HCl secretion was seen (fig. 2).

In other experiments we investigated the effect of acetylcholine on the peptic secretion. A gastric secretion was evoked by continuous intravenous injection of histamine. When the juice became pepsin free acetylcholine was concomitantly given. Experiments were made on eserinizd as well as on non eserinizd ani-

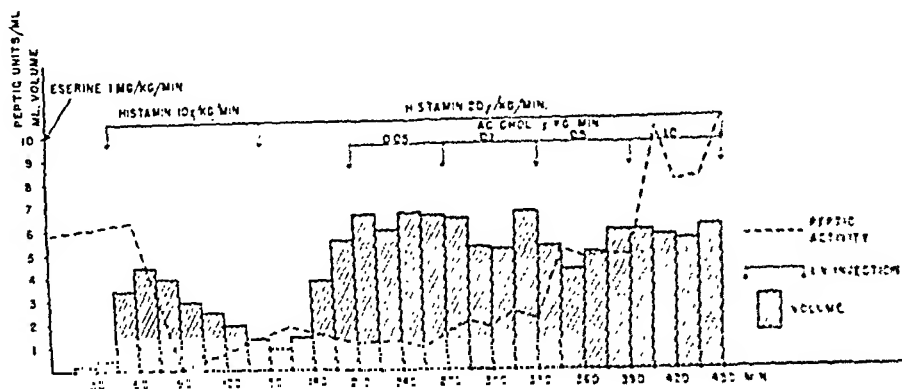


Fig. 4. Cat 3.1 kg. The secretion of pepsin in an eserinizd cat under intravenous injection of acetylcholine. Histamine concomitantly given slowly intravenously.

mals. In a few experiments a small increase of the peptic activity was seen in eserinizd cats. (Fig. 4.)

In a few experiments (5) we investigated the secretory effect of doryl. Doses were tried from 0.001 γ per kg body weight to toxic amounts. No significant HCl or peptic secretion was observed.

Di-isopropyl-fluorophosphate. Di-isopropyl-fluorophosphate (DFP) causes in vitro and in vivo a pronounced inhibition of the enzymatic destruction of acetylcholine. Its parasympathetic effects are considered to be due to the accumulation of acetylcholine in the tissues caused by this inhibition. MOORELL et al. (1946) studied the effect of DFP on cats. After the administration of 0.5–2 mg DFP intravenously, amounts which strongly inhibit the enzymatic destruction of acetylcholine, among other effects they observed a copious salivary secretion and an increased gastrointestinal motility. In our experiments DFP was given intramuscularly. 2–5 mg per kg evoked a copious secretion of an acid gastric juice. Fig. 5 shows this secretory response to DFP. As seen it is very similar to that after eserine. The secretory effect was completely abolished by atropine 1 mg per kg body weight.

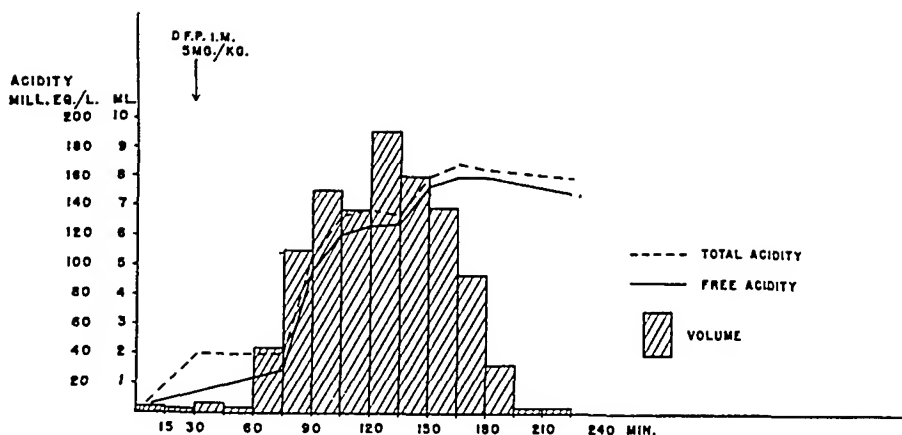


Fig. 5. Cat 3 kg. Secretory response to di-isopropyl-fluorophosphate 5 mg/kg i. m.

Discussion.

Atropine has been shown to inhibit the HCl secretion induced by vagal stimulation, although the doses necessary to completely block the secretory response are rather high. Eserine stimulates the HCl as well as the peptic cells. The point of attack is peripheral as the secretory effect remains after vagotomy and splanchnicotomy. It is abolished by atropine. As the doses of eserine given probably were adequate to cause a considerable inhibition of the enzymatic destruction of acetylcholine it is likely that the secretory effect in part at least is due to the accumulation of acetylcholine in the gastric mucosa. This assumption is strengthened by the fact that DFP, the parasympathetic effects of which are attributed to its inhibitory action on choline esterase, in doses adequate to be considerably inhibitory evokes a copious gastric secretion. This secretion is abolished by atropine.

The experimental evidence presented in this paper favours the view that acetylcholine is involved in the mediation of vagal secretory impulses to the gastric cells. Why is it then impossible to evoke any HCl secretion by the intravenous or intraarterial administration of acetylcholine? In our experiments acetylcholine was given to eserinated, and non eserinated animals in such a wide range of doses that in some experiments at least, acetylcholine, if a secretory stimulant, would have been expected to reach the parietal cells in a concentration adequate to activate the secretory mechanism.

Experimental evidence indicates that the mechanism of HCl

secretion is a complicated one. BABKIN has in several papers brought forward the idea that histamine in one way or another is involved in the activation of the HCl cells in the cephalic phase of the gastric secretion. MACINTOSH (1938) found histamine in the gastric juice obtained after sham feeding, electrical stimulation of the vagi and subcutaneous injection of histamine. EMMELIN and KAHLSON (1944) showed the existence of active histamine in the gastric juice secreted in response to electrical stimulation of the vagi, sham feeding, a meal, gastrin and histamine. They consider their results favour the view that histamine acts as a humoral transmitter (possibly local) whenever an HCl secretion is evoked. NECHELES and co-workers reported that acetylcholine and histamine act as synergists. UVNÄS (1942) observed in acute experiments on cats that the secretory response to electrical vagal stimulation disappeared or was markedly depressed when the pyloric mucosa was anaesthetized with cocaine or otherwise functionally disconnected. The secretory response to gastrin was, under similar conditions, found to be abolished or markedly diminished. However, during concomitant vagal stimulation and administration of gastrin a copious gastric secretion was observed.

The experimental facts cited above indicate that the secretion of HCl can be enhanced by the concomitant action of different secretory influences. The failure to evoke any HCl secretion by the administration of acetylcholine might be due to the fact that this substance either does not penetrate into the secretory cells or that it is only able to activate these cells in combination with other secretory agents. The combined action of two or several humoral transmitters might be necessary to activate the secretory mechanism (BABKIN 1944).

The slight increase of the pepsin secretion observed under the concomitant administration of acetylcholine and histamine indicates that acetylcholine is involved in the activation of the pepsin secretory mechanism. The part played by histamine in this process is obscure. The neutral or slightly alkaline mucous juice obtained after acetylcholine is pepsin free. We agree with STAVRAKY that the lack of enzymatic activity probably is not due to the rapid inactivation of secreted pepsin. In our experiments the collected mucous juice was immediately acidified to prevent such a destruction. The fact that pepsin appears in the gastric juice only if histamine is concurrently given does not however necessitate the assumption that histamine is involved in the intimate

secretory process of pepsin. As pointed out by BABKIN the anatomical arrangement of the gastric glands favour a stagnation of the mucous secretion from the peptic glands in the glandular tubules. The effect of histamine might simply be to produce a watery HCl secretion, that washes out the formed pepsin.

Summary.

In chloralozed cats:

Atropine inhibits the gastric secretion evoked by electrical stimulation of the vagi.

Eserine stimulates the HCl as well as the peptic cells.

DFP stimulates HCl secretion.

Acetylcholine given intravenously or intraarterially in eserized as well as non eserized animals does not induce any HCl secretion. It stimulates only the peptic and the mucous glands.

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Is the Secretion of Pepsin Hormonally Controlled?

By

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It is an established fact that vagal impulses activate the pepsin glands. In addition old observations by members of the Pavlov group indicate that the pyloric region plays a part in the regulation of the peptic output in the stomach. ZELJONY and SAVICH (1914) and SAVICH (1922) claimed that mechanical and chemical stimulation of the isolated pyloric pouch of a dog increased the concentration of pepsin in the juice secreted by the glands of the corpus and the fundus.

PRATT (1940) reported that the intravenous injection of secretin on chloralosed cats produced peptic activity in the pepsin free gastric juice obtained by subcutaneous injection of 1 mg histamine. PRATT does not announce the degree of purification of his secretin preparations but reports that identical results were obtained with a commercial product prepared according to ÅGREN (possibly pancreotest Astra, Sweden). As gall salts instilled into the duodenum were also observed to augment the pepsin concentration PRATT concludes that the secretin molecule stimulates the peptic cells. Later in similar experiments on cats BABKIN and KOMAROV (1944) showed that an increase of the pepsin output occurred only if impure secretin preparations were used. The so called S1 material prepared according to GREENGARD and IVY (1938) lost its pepsigogue effect on purification. Crystallized secretin was devoid of any pepsigogue action.

GROSSMAN et al. (1944) studied the pepsin content of gastric

juice "secreted in response to hormonal stimulation". Vagotomized total stomach pouches on dogs were perfused with histamine free liver extracts. A gastric secretion of low peptic activity was observed in the subcutaneously transplanted pouches of these dogs. The peptic output was of the same low order as that observed after subcutaneous doses of histamine. The authors conclude that as regards pepsin histamine could be the humoral agent responsible for the type of secretion obtained. This opinion is in accordance with that of the Ivy group, as presented in the paper of BUCHER, IVY and GRAY (1941). These authors consider histamine as a pepsin stimulating agent on dogs. The evidence presented by BABKIN and collaborators (BOWIE and VINBERG (1935) and others) as well as from this laboratory by BJÖRKMAN et al. (1943) indicates that histamine has no pepsinogenic effect on cats or dogs.

Supporting evidence for the existence of a hormonal control of the secretion of pepsin was obtained by FRIEDMAN et al. (1944). They observed on PAVLOV pouch dogs, secreting in response to insulin or food, that instillation of HCl into the small intestine increased the pepsin content of the gastric juice.

Substances stimulating the enzyme secretion of the digestive glands have been isolated in several laboratories. HARPER et al. (1943, 1945, 1947) have obtained from the duodenal mucosa an agent, pancreozymin, that increases the enzymatic output of the pancreas on cats. GREENGARD and IVY (1945) working on dogs confirmed the existence of pancreozymin and separated it from secretin (GREENGARD 1947). NASSET and his group isolated from the intestinal mucosa of dogs and pigs another substance, enterocrinin, that according to FINK (1941) with a dose of 36 γ evokes an accurately measurable secretory response of the small intestinal mucosa. The volume as well as the enzymatic output is augmented.

HARPER as well as NASSET, when presenting their papers at the International Physiological Congress in Oxford this year emphasized their opinions that the enzyme secretion of the pancreas and the glands of the small intestine is hormonally controlled, pancreozymin and enterocrinin being the respective hormonal agents.

In a previous paper UVNÄS (1945) reported that some of his extracts from the pyloric mucosa in addition to their stimulating effect on the HCl secretion evoked a slight increase of the peptic

output. These observations have been confirmed and extended in later experiments. Owing to the increased interest in hormonal mechanisms regulating the enzyme production of the digestive glands it seemed justifiable to publish the results although the experiments are not yet complete.

A. Experiments with Pyloric and Duodenal Extracts.

The experiments were performed on vagotomized cats under combined chloralose-urethane anaesthesia (50 mg chloralose, 500 mg urethane per kg). A pepsin free gastric secretion was induced and maintained by slow intravenous injection of histamine. The gastric juice was collected from a cannula inserted into the stomach and pushed out through a stab wound in the abdominal wall. The acidity of the juice was determined by titration with N/10 NaOH; phenolphthalein was used as indicator. The peptic activity was measured by the method of ANSON and MIRSKY (1936), slightly modified. Extracts from the pyloric and duodenal mucosa of cats, dogs and pigs were prepared according to MUNCH-PETERSEN et al. (1944) and Uvnäs (1945) and injected intravenously.

Results.

Earlier observations were confirmed that impure preparations obtained from HCl extracts of the pyloric mucosa by precipitation with sodium chloride, tannic acid and especially trichloroacetic acid induced a slight increase of the peptic activity concurrently with their stimulating effect on the HCl secretion. The acidity of the gastric juice obtained amounted to about 150—160 mill. eq./l. Washing the active material with 80 % ethyl alcohol (MUNCH-PETERSEN et al.) or isoelectrical precipitation (Uvnäs), both processes that augment the secretagogue effect of the material on the HCl glands, diminished the pepsinogenic properties. Fig. 1 a and b show the increase of the pepsin concentration after the injection of three different extracts prepared by different methods. The materials were given in doses approx. equal in their power to stimulate the HCl glands. No correspondence was observed between the secretory response of the HCl and the pepsin glands to the different extracts.

Impure preparations of duodenal mucosa from dogs and pigs were observed to evoke a slight increase of the peptic activity, as did the commercial Swedish secretin product Pancreatost

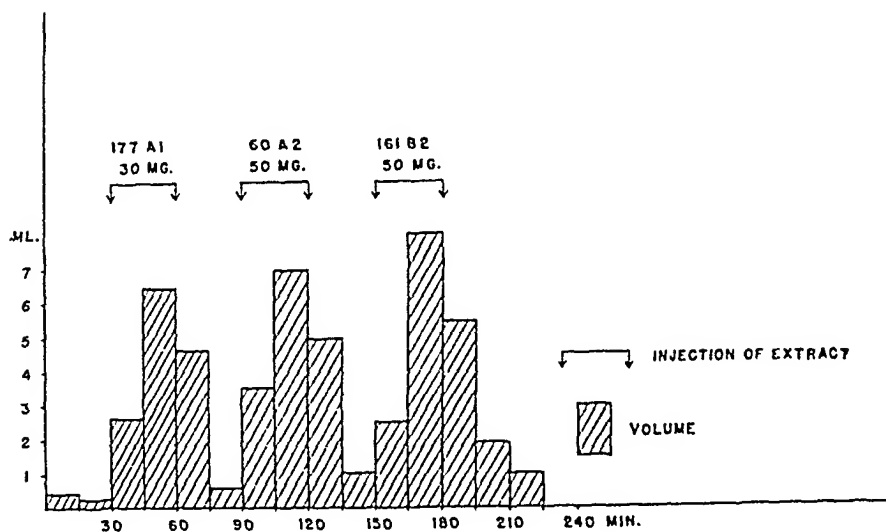


Fig. 1 a. Cat. 3.9 kg. Chloralose-urethane. Volume of the HCl secretion evoked by three different pyloric preparations. Prep. no. 177 a₁ (from cat) precipitated by trichloroacetic acid (MUNCH-PETERSEN et al. 1944) Prep. no. 160 a₁₁ (from pig) prepared by the tannic acid method (MUNCH-PETERSEN et al. 1944). Prep. no. 161 b₁₁ (from pig) prepared by the trichloroacetic acid method (MUNCH-PETERSEN et al. 1944).

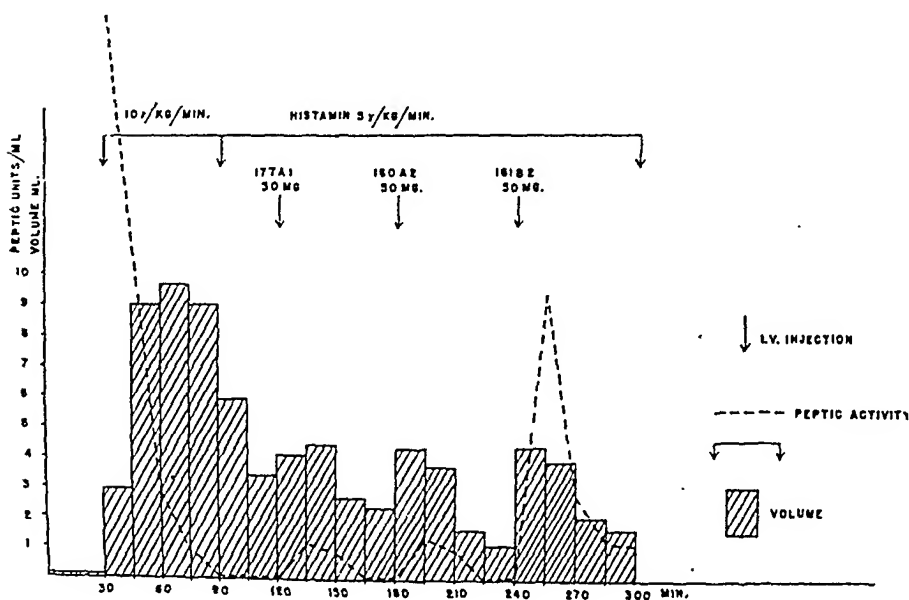


Fig. 1 b. Cat 3.4 kg. Chloralose-urethane. Peptic output evoked by three different pyloric preparations (the same as in fig. 1 a). A basal secretion of HCl was initiated and maintained by intravenous administration of histamine.

(Astra). No experiments were performed with crystalline secretin preparations.

A circumstance rendering the investigation of the pepsigogue properties of the extracts difficult was the fact that the animals varied considerably in their pepsin response. The reason for this variation is obscure but as we obtained our cats from different sources the irregularities may have a dietary basis. It was reported from Babkin's laboratory that on dogs the pepsin secretion varied with the diet. (CAMPBELL 1934, MACINTOSH and KREUGER 1938.)

B. Attempts to Elicit Pepsin Secretion by Stimulating the Pyloric and the Duodenal Mucosa.

As the pepsigogue effect of pyloric and duodenal extracts indicated the possible existence of a hormonal mechanism playing a part in the regulation of the pepsin secretion a series of experiments were made on cats and dogs to evoke a pepsin secretion by the stimulation of the duodenal and pyloric mucosa.

Experiments on cats.

We were unable to verify Pratts report that gall salts in the duodenum stimulate the pepsin secretion. The sodium salts of taurocholic as well as glychocholic acid were instilled into the duodenum of anaesthetized cats but no pepsigogue effect was observed.

Experiments on dogs.

On dogs with a gastric and a duodenal fistula we investigated the effect of mechanical stimulation of the pyloric mucosa on the pepsin secretion. A rubber balloon was inserted through the duodenal cannula and placed in the pyloric part of the stomach. When inflated with air the balloon distended the pyloric region and at the same time obstructed the passage from the stomach to the duodenum. This procedure elicited in some dogs a profuse secretion of a gastric juice that was highly acid but rather poor in pepsin. To decide whether the peptic activity of the juice was due to an activation of the pepsin glands or only secondary to a "washing out" of preformed pepsin by the HCl secretion, the gastric glands were in other experiments activated by repeated subcutaneous doses of histamine. When the peptic activity of the juice was expected to be practically lost the rubber balloon was placed in the duodenum and inflated.

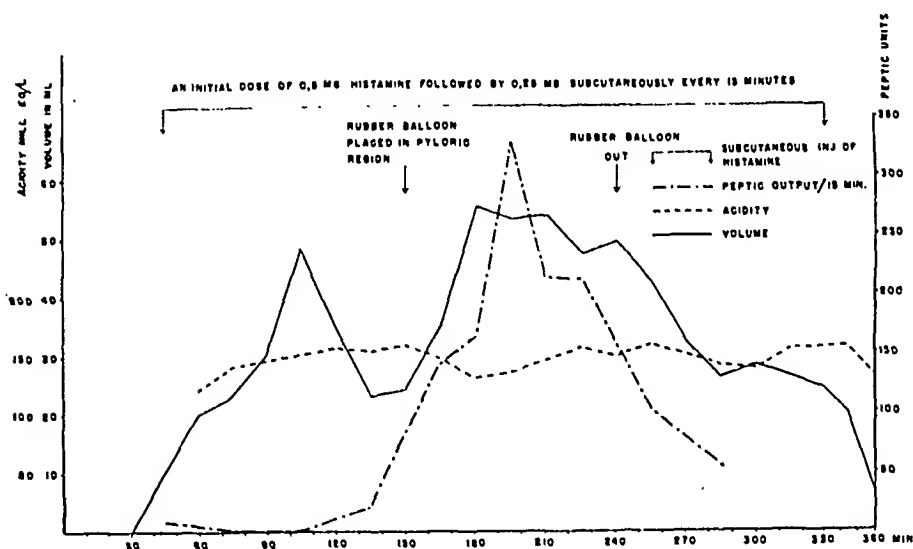


Fig. 2. Dog 16 kg, with one gastric and one duodenal fistula. The peptic (and HCl) output elicited by mechanical stimulation (distension) of the pyloric region. A basal pepsin free gastric secretion was maintained by repeated subcutaneous doses of histamine.

Fig. 2 illustrates one of these experiments. An initial dose of 0.5 mg histamine was given subcutaneously and followed by 0.25 mg every 15 minutes. The pepsin output that before the stimulation of the pyloric mucosa is very low, increases considerably when the balloon is inserted and inflated. It rapidly declines when the balloon is removed. The HCl secretion is markedly increased during the same period. It seems reasonable to refer the secretory response of the pepsin as well as the HCl glands to the mechanical stimulation of the pyloric mucosa.

Discussion.

The occurrence of a pepsigogue factor in extracts from the pyloric and duodenal mucosa is a very interesting fact as from these mucosal regions other secretagogue principles have been obtained believed to be identical with physiologically occurring hormones such as gastrin, secretin, pancreozymin and enterogastrone. The new pyloric principle is not identical with gastrin, as this substance selectively stimulates the HCl glands (KOMAROV 1942, UVNÄS 1945, HARPER 1947). The pepsigogue factor in duodenal extracts is not identical with secretin as shown by BABKIN and KOMAROV (1944) and BUCHER and GREENGARD (1942). The pepsin

stimulating effect cannot be ascribed to histamine as this substance does not occur in the secretin preparations used by BABKIN and KOMAROV or the gastrin preparations used (UVNÄS). No attempts have been made in the present study to investigate the chemical properties of the pepsigogue principle but to judge from the chemical behavior of the agent it seems to be of a structure, related to the other secretagogue principles isolated from the pyloric and duodenal mucosa. To judge from the findings of BABKIN and KOMAROV the pepsigogue principles from the pyloric and duodenal mucosa could be identical.

Mechanical stimulation of the stomach mucosa is reported by several investigators to activate the gastric glands. Lately GROSSMAN (1947) has shown that this activation is mediated by a hormonal mechanism. He observed gastric secretion from a subcutaneously transplanted pouch stomach when the main stomach was distended by a balloon. To judge from older observations by ZELJONY and SAVICH (1914), SAVICH (1922), CHANG and LIM (1931) and others the region chiefly responsible for the secretion elicited by mechanical stimulation is the pyloric mucosa. In our experiments the mechanical irritation has been exerted on the pyloric mucosa even if it probably has not been entirely confined to this region. No definite conclusion can be drawn concerning the intimate mechanism involved in the activation of the peptic glands in these experiments. It cannot possibly be due to the absorption of a secretagogue agent. Theoretically it is possible that mechanical stimulation may stimulate "mucosal receptors" and the peptic cells be activated by impulses transmitted over some reflex arcs. Such nervous structures are not known to exist however. Against the background of our present knowledge of the different hormonal mechanisms regulating the activity of the digestive glands, and the recent findings of GROSSMAN and IVY mentioned above, it seems most logical to assume that the pepsin secretion elicited by mechanical stimulation of the pyloric mucosa is mediated by a hormonal mechanism.

The observations that a pepsigogue principle can be obtained from pyloric extracts and that the pepsin glands can be activated by mechanical stimulation of the pyloric region together with the fact that the enzymatic secretion from other digestive glands is supposed to be hormonally controlled make it reasonable to assume that the pepsigogue pyloric principle is a physiologically occurring secretagogue.

Summary.

A pepsigogue principle is observed in extracts from the pyloric and duodenal mucosa of cats, dogs and pigs.

The peptic cells are activated by mechanical stimulation of the pyloric mucosa.

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Investigations
on
the Structure and Function of Living, Isolated,
Cross Striated Muscle Fibres of Mammals

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From the Laboratory for the Theory of Gymnastics, University of Copenhagen

Investigations
on
the Structure and Function of Living, Isolated,
Cross Striated Muscle Fibres of Mammals

By
Poul Høncke

AARHUS

1947

Denne Afhandling er af det lægevidenskabelige
Fakultet antaget til offentlig at forsvares for den
medicinske Doktorgrad.

København, den 24. Juni 1947.

Mogens Fog,
h. a. dec.

PREFACE.

The experiments for the present investigation were carried out in the Laboratory for the Theory of Gymnastics, University of Copenhagen, during the years 1939—1943. I wish to express my sincere thanks to the chief of this laboratory, Professor *Emanuel Hansen*, D. Sc., for the excellent working facilities afforded me at the laboratory.

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Poul Høncke.

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INTRODUCTION.

In spite of the numerous histological investigations carried out on cross striated muscular tissue conflicting views still prevail on many points — even as regards the mere description of the microscopical image of the cross striated muscle fibres at rest.

The reason for the conflicting observations should in the first instance be sought in the complicated structure of the muscle fibres. The diffraction of the muscle fibres may cause erroneous optical images, and some of their structural elements are just microscopically discernible — a fact which may give rise to differing interpretations. Fixing may produce rather pronounced alterations in the structure of the muscle fibres for which reason fixed preparations should not be used for histological investigations except with suitable reservations.

Several authors have emphasized these sources of error (i. a. *Engelmann*, 1873, *Hürthle*, 1909 and *Buchthal*, *Knappeis & Lindhard*, 1936), but the great majority has paid no regard to the warnings — exclusively using fixed material for investigations on the structure of muscle tissue — they have even described the structural changes during contraction on the basis of fixed material.

Investigations of fixed preparations should not, however, be completely rejected, as it is impossible or, at any rate, difficult to recognize some of the structural elements, as e. g. the nuclei, in living muscle fibres, while they are clearly seen in fixed, stained preparations.

There are other methods than the purely microscopical examination available for the analysis of the structure of muscle fibres; the following may be mentioned: investigations on the optical properties of the muscle fibres, especially their function as a diffraction grating, investigations on birefringence, roentgen-spectroscopical investigations, investigations on the mechanical and thermo-elastic properties of the muscle

fibres and finally electro-physiological investigations; but also in these fields it holds that living, isolated fibres should be used and not fixed preparations of whole muscles.

From the Laboratory for the Theory of Gymnastics, University of Copenhagen, a series of investigations on the structure and function of muscle have been issued in which allowance has been made for the above mentioned sources of error and in which the experiments have been performed on living isolated muscle fibres of frogs under well-defined experimental conditions. The present work should be regarded as an integral part of this series, the author having attempted to perform a similar analysis of cross striated muscles from mammals, especially in order to correlate structure and function and to ascertain the extent of consistency between striated muscle fibres from frogs and mammals; in other words to investigate whether the principles which apply to the structure and function of muscle fibres from frogs also hold with regard to mammalian muscle fibres.

The investigation will thus comprise: an introductory summary of the literature on the topic, dealing preferably with investigations performed on living non-fixed preparations; a short description of the alterations which may be produced by fixing and staining the muscle fibres; selection of suitable experimental material; elaboration of a convenient technique for isolating living mammalian muscle fibres and for securing the survival of the latter; histological examination of the preparations thus made, by direct microscopical observation and by means of microphotography. The height of the muscle compartment and that of the isotropic and anisotropic layer has been measured on the microphotographs, and the muscle fibres have been examined at various degrees of stretch both at rest and during contraction; examinations of various muscles of the guinea pig and orientating examinations of muscle fibres from other mammals: rabbit, cat and goat have been performed. The structural changes during contraction have furthermore been recorded as a function of time. The birefringence of the muscle fibres have been measured and the mechanical properties of the muscle fibres have been studied to illustrate the minute structure and its function. In order to facilitate comparison the experimental material has, as far as possible, been treated in the same manner as in the experiments on muscle fibres of the frog.

CHAPTER I.

PREVIOUS INVESTIGATIONS.

This chapter will deal with previous investigations of the structure of muscle tissue. It is in this paper the intention only to mention investigations carried out on living muscle fibres and especially those in which the structure is correlated to the function.

A short summary of the structure of the muscle tissue is given as an introduction in order to establish the nomenclature employed. In the following sections the individual structural elements will be discussed and so will — for practical reasons — the author's own observations of living mammalian muscle fibres. A fairly thorough summary is given of previous investigations on the variations of the cross striation during contraction, and special emphasis is also laid on investigations which elucidate the minute structure of muscle tissue. Finally the chemical and physical changes which may be caused by fixing are discussed — the discussion being illustrated by own experiments — in order to substantiate why only living muscle fibres have been used in the present investigation.

A. Summary of the Structure of Muscle Tissue.

Muscles are made up of muscle fibres and connective tissue. The muscle fibre is the histological unit of the muscle. For defining the length of a muscle fibre the equilibrium length is used, i. e. the length at which the tension of the muscle fibre is zero, but at which a tension is set up by even the smallest elongation. The elongation of the fibre is expres-

sed as a function of the equilibrium length which is put = 100

$$\left(\frac{\text{length (x)}}{\text{equilibrium length}} \times 100 \right).$$

The muscle fibre is enclosed by an elastic sheath, sarcolemma, which consists of a fine collagenous network of fibrils embedded in a homogeneous substance.

The contractility of the muscles is caused by longitudinal structural elements of the muscle fibre — the myofibrils — which consist of alternate isotropic and anisotropic layers, identical layers in the various myofibrils being held at the same level throughout the fibre, a fact which accounts for the cross striated appearance of the fibre.

The birefringent layer is known as A (= anisotropic substance, Q = Querscheibe, sarcous substance, disque transversale, disque epais ou sombre) while the monorefringent layer is known as I (= isotropic substance, hyaline substance, disque claire). In ordinary light the isotropic layer appears light, the anisotropic dark, but if the muscle fibres are examined in polarized light between crossed nicols the opposite is the case. In stained preparations the A-layer is usually stained most intensely.

In the middle of the I-layer there is a fine transverse structural element Z (= Zwischenscheibe, Krause's membrane, Lealand's stripe, Dobie's line, telophragma, disque mince, strié d'Amici) which is connected with sarcolemma and which is assumed to keep the myofibrils in place.

The segments between two Z-membranes are collectively known as a muscle compartment (sarcomere).

It is doubtful whether the following terms denote actual structural elements: Q_H (= Hensen's or Dobie's stripe) which is seen as a lighter band in the middle of the A-substance, M (= Mittelscheibe, mesophragma, Mittelmembran (Heidenhain)), which is seen as a fine, dark, transverse line in Q_H, and, finally, N. (= Nebenscheiben) which appear as narrow, dark cross stripes on both sides of Z at a slight distance from the latter.

Between the longitudinal structural elements of the muscle fibre there is a liquid substance, the sarcoplasm, which besides salts and colloid particles contains the pigment of the muscle, the myoglobin.

Finally the muscle fibre contains numerous nuclei which in mammalian muscles are situated below the sarcolemma,

while in the muscles of frogs they are also found in the interior of the muscle fibres.

In the muscle the individual fibre is encased in a fine network of connective tissue, the endomysium. By connective tissue, known as the perimysium internum, the fibres are arranged in primary bundles, the fascicles, which are in turn held together in larger bundles by coarser connective sheaths while the whole muscle is surrounded by a layer of connective tissue, the perimysium externum, or muscle fascia, of varying thickness.

A motor unit denotes a motor nerve cell and the 50—300 muscle fibres which it innervates.

B. The Macroscopic and Microscopic Structure of Muscle Tissue.

The Shape and Size of the Muscle Fibres.

Shape.

Muscle fibres may extend from the origin of a muscle to the insertion and in that case they have blunt or conical endings (*Bardeen*, 1903), while muscle fibres with free, intra-fascicular ends (*Rollett*, 1856) taper towards the ends, the fibres being fusiform (*Herzig*, 1858, *Weber*, 1851), lanciform or whipshaped (*Lindhard*, 1926, p. 200—203). Ramified muscle fibres with free endings have been observed — in the membrana basohyoidea of the frog (*Fischel & Kahn*, 1928, *Nagamitu*, 1932).

It is to a certain extent illusory to speak of the shape of the cross section of a muscle fibre as the fibres are rather plastic. In vivo the cross section of the muscle fibre is circular, perhaps slightly flattened on account of mutual pressure. By measuring the diameters of isolated, freely suspended muscle fibres from m. semitendinosus of the frog in two planes at right angles to each other it was possible for *Buchthal & Knappeis* (1946) to show that the cross section of the fibre is usually somewhat oval even under these conditions when the fibres are not exposed to external mechanical stresses. Only 17 per cent of the 105 preparations measured by the above mentioned method had a circular cross section, in 36

per cent there was a difference of up to 10 per cent between the two diameters, while only in 13 per cent the difference between the diameters exceeded 40 per cent.

Size.

As the area of the cross section is not the same throughout the whole length of the fibre the thickness of the fibre should not be determined on the basis of only one cross section. This source of error is e. g. met with in pathology when muscle biopsy is employed for diagnosing certain types of atrophy (*Schimert, 1934; Wohlfart, 1935*).

In m. gastrocnemius of *Rana temporaria*, but not of *Rana esculenta* *Lindhard* (1926, p. 127) found muscle fibres of two different thicknesses with an average diameter of 96 μ and 44.5 μ respectively, and the fibres were arranged in pairs consisting of a thick and a thin fibre. In man the primary muscle fibre bundles contain, according to *Wohlfart* (1935), fibres of 2—3 different sizes, and he assumed that fibres of the same size are innervated by the same motor nerve cell, i. e. belong to the same motor unit. In a more comprehensive investigation (1937) *Wohlfart* has tried to show that in man and various mammals each primary bundle of muscle fibres contains 1—2 special fibres, called b-fibres, which are larger than all other fibres (a-fibres), and which presumably have an unknown, specific function. Such fibres may be observed particularly clearly in certain muscles, e. g. the external muscles of the eye, while in other muscles they can only be seen in embryos and in newborn individuals and in case of muscle atrophy, e. g. senile atrophy.

In the same individual the thickness of the fibres in the various muscles is to a certain extent the same; *Voss* (1934—37) thought it possible to show that the finer the movements a muscle is able to perform the smaller the cross section of the fibres. The muscles fibres of the external muscles of the eye are particularly fine, while coarse fibres are found e. g. in the long dorsal muscles and in the mm. glutei (*Mayeda, 1890*). Within the various animal classes the thickness of the fibres varies (*Mayeda, 1890*); fishes and amphibians have the thickest and most varying fibres while mammals and especially birds have thinner and more uniform fibres.

In man the thickness of the muscle fibres is stated to

The Cross Striation of the Muscle Fibres.

In the middle of the last century *Bowmann* (1840) described how muscle fibres may be split both longitudinally in fibril-fascicles and transversely in discs, resulting in what he called »sarcous elements«; *Lealand* (1848) and later on *Amici* (1859) and *Krause* (1868) described the Z-membranes; *Dobie* (1849) and *Hensen* (1868) described Q_H , and *Brücke* (1858) observed the anisotropy of the A-layers, *Flögel* (1872) described N.

According to *Engelmann* (1873) the cross striation of the muscle fibres is very complicated, consisting of A-, I-, Z-, N-, Q_H - and M-cross striae, while i. a. *Krause* (1873) was of the opinion that in vivo the muscle fibres only exhibit A-, I- and Z-cross striae, and *Hürthle* (1909) has even denied the in vivo existence of Z. The discussion about the cross striation of muscle fibres has been continued since then. In the following only the most important observations are to be mentioned, while further reference may be made to summaries by *Heidenhain* (1911), *Häggquist* (1931), *Jordan* (1933), *W. J. Schmidt* (1937) and *Buchthal & Lindhard* (1939).

A and I.

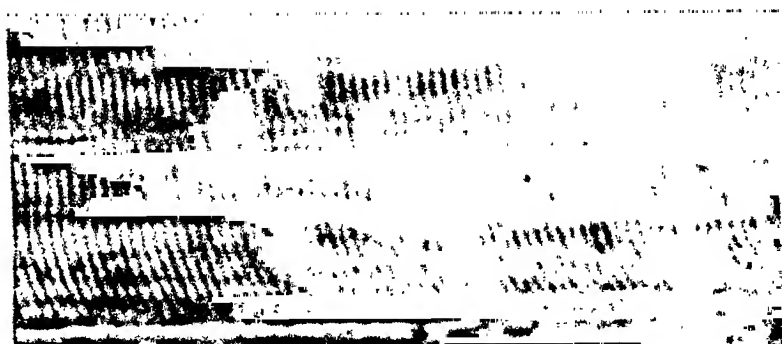
It is generally agreed that there are actual A- and I-cross striae of the muscle fibres, this has also been found by examination of living muscle fibres (figs. 1—2). It is likewise generally acknowledged that the A- and I-cross striae are due to periodical differences in the refraction of the fibrillar structure; this has i. a. been shown by electron microscopical examinations of myofibrils (*Hall et al.*, 1946).

In his model of the minute structure of the myofibrils *Hürthle* (1931, b) explains the difference between the anisotropic and isotropic segments of the myofibrils as caused by different orientation of the micellar chains of the elementary fibrils. In the A-segments the arrangement of the micellar chains is parallel — i. e. crystalline — for which reason the myofibrils are birefringent, in the I-segments the arrangement of the micellae is more irregular.

The I-segments were previously assumed to be isotropic. *W. J. Schmidt's* investigations (1934, 1935) proved that the myofibrils are anisotropic throughout their length, also in the I-segments, the birefringence of the I-segments being 10 times



a



b



c

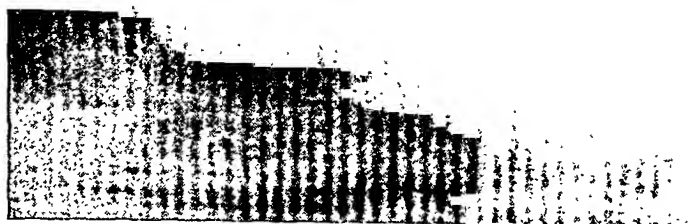


d

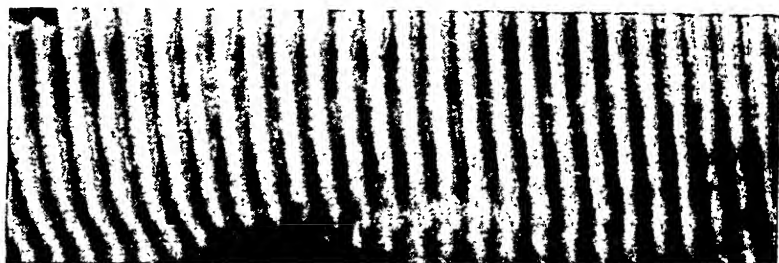
Fig. 1.

Muscle fibres from m. gluteus max. of the guinea pig

- a) Fibres at equilibrium length, showing slight relative displacement of the fibril bundles. Magnification 250 \times .
- b) Stretched muscle fibres. Height of compartment 2.2 μ . Showing antitrople layers in neighbouring fibres to be on the same level. Magnification 400 \times .
- c) Stretched muscle fibre at rest. Height of compartment 2.0 μ . Magnification 1100 \times .
- d) Same fibre during isometric tetanic contraction. Height of compartment 2.8 μ . Magnification 1100 \times .



a



b

Fig. 2.

Muscle fibres from m. gluteus max. of the guinea pig.

- a) Muscle fibre at equilibrium length between crossed nicols. A appears light, I dark. Magnification 750 \times .
- b) Muscle fibre at equilibrium length between crossed nicols. The Z-membrane manifests itself as a clearing up in A. Magnification 1400 \times .

lower than that of the A-segments. If *Hürthle's* model is considered in the light of this observation it means that the micellar chains, even in I, have a certain longitudinal orientation. According to *d'Ancona* (1930) the myofibrils are of uniform chemical nature and birefringent in the whole of their length, the gradual differences in the anisotropy of the muscle is due to isotropic I-granules, varying content of water and varying density of the myofibrils. *Liang* (1936) was likewise of the opinion that the myofibrils are birefringent throughout their length and that the cross striation is caused by a perifibrillar, isotropic substance of a netlike structure.

Finally we may mention a few conceptions of the muscular structure which are rather different from the above mentioned: *Marcus* (1920—1927): The fibrils are tubular elements, the cross striation is due to deposits on the fibrils. *Lutembacher* (1928, pp. 19—28): The cross striation is caused

by the myofibrils having a corrugated or fluted surface. *Lange-laen* (1928, 1936): The muscular structure is build up of a system of lamellae. *Carey* (1936—1940): The cross striation is "alternate places of condensation and rarefaction of a standing system of pressure waves transmitted through protoplasm".

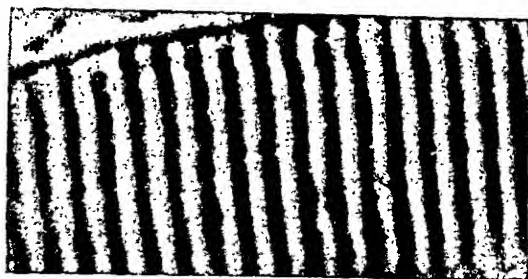
The A- and I-cross striae are further dealt with in section C of this chapter.

Z.

There are only a few authors who deny the vital existence of the Z-membranes (*Hürthle*, 1909, 1931, c; *Boerner-Patzelt*, 1929; *Ernst & Kellner*, 1936, b). In living muscle fibres they have been observed by i. a. *Meigs* (1908), *d'Ancona* (1930), *Jordan* (1934), *Studnitz* (1935, a), *Buchthal*, *Knappeis & Lindhard* (1936), and *Speidel* (1938, 1939). *v. Muralt* (1933) could always recognize the cross membranes when he examined the fibres in ultraviolet light. In the ashes from frog fibres after micro-incineration *Buchthal & Lindhard* (1939, p. 31) observed distinct Z-stripes. In fixed and stained preparations the Z-membranes are always to be seen, here they often appear as the most distinct cross striae. The Z-membranes are anisotropic — they appear light in polarized light.

In living muscle fibres from mammals Z-stripes are not seen in all preparations. As these structural elements are so fine a special illumination is required to make them appear (figs. 2—3). They are more clearly seen in extended than in relaxed fibres, presumably because the cross-sectional area of the fibre decreases when the fibre is extended so that the structure becomes more "dense". Contraction, however, do not increase the visibility of the Z-membranes. The thickness of the Z-membrane does not seem to be constant, but it is not possible to lay down any proportionality between the thickness of Z and the degree of stretch of the fibre, as maintained by *Engelmann* (1873). The optic image of the thickness of the Z-membrane is also a function of the depth of focus of the objective and the orientation of the membrane in relation to the optical axis of the objective.

Considerable disagreement prevails with regard to the structure and function of Z. According to *Hägglquist's* opinion (1920, b) force is transmitted from the contractile myofibrils



a



b

Fig. 3.

Muscle fibres from m. gluteus max. of the guinea pig.

- a) Slightly stretched muscle fibre showing Z-membrane and Q_H (top left part of the photo). On account of altered focusing the ratio $A:I$ appears abnormal. Magnification $1400\times$.
- b) Muscle fibres exhibiting false M-cross striae, produced by oblique illumination and defective focusing. In the right part of the photo Z can be seen centrally in I. To the left Z can be seen forming part of the dark cross striae. Magnification $1000\times$.

to the connective tissue of the muscle via the Z-membranes. The latter consist of collagenous substance and they divide the fibrils in segments and are intimately connected to sarcolemma and endomysium. According to *v. Møllendorff* (1925), however, the staining methods used by *Häggquist* are not specific to collagen. Electron microscopical examinations (*Hall et al.*, 1946) have shown that the Z-membranes are not collagenous. Most investigators agree that there is an interfibrillar Z-structure attached to the sarcolemma, it can e.g.

be seen when the fibres appear festooned with sarcolemma retracted corresponding to the Z-membranes (Meigs, 1908; Jordan, 1934). There is evidence that the Z-membranes continue through the myofibrils. By treating the muscle fibres with a potassium hydroxide solution of suitable concentration it is possible to make the myofibrils disintegrate into cross discs — *Bowmann's discs*. *Buchthal's & Péterfi's* (1934) electrostatic measurements of living isolated fibres indicated that the potential difference is proportionate to the number of muscular compartments between the two electrodes, and this was considered to be inconsistent with a continuous fibrillar structure. The potential difference will also decrease as soon as Z is destroyed. A fenestration of the Z-membrane has never been observed microscopically, and by means of the electron microscope the material of the Z-membrane can be observed between the myofibrils, cementing them together and penetrating through them (Hall et al., 1946). *Apathy* (1907) thought that the nature of Z must differ according to its occurrence intra- or interfibrillarly, as it is possible, e.g. by boiling, to make the muscle fibres disintegrate longitudinally, corresponding to the fibrils, without a simultaneous disintegration into *Bowmann's discs*.

On one hand the Z-membranes must be elastic as their areas vary considerably according to the degree of extension of the muscle fibres, on the other hand it is — as emphasized by *Lindhard* (1926, p. 213) — most probable that *Häggquist's* theory is correct and that the contraction tension is transmitted via the Z-membranes to the connective tissue, but this is inconsistent with the idea of high elasticity. It is also held that the Z-membranes maintain the orientation of the myofibrils in such a way that the isotropic and anisotropic segments of the myofibrils are kept in the same cross-sectional plane. By stretching the muscle fibres, however, displacement of the myofibrils can be observed, and this displacement is reversible. None of the theories hitherto propounded with regard to the Z-membranes can fully explain these phenomena.

Q_H .

The fact that Q_H occurs in vivo has e.g. been verified by *Stübel* (1920) and *Studnitz* (1935, a). Q_H has been assumed to be a refraction phenomenon, *Exner* (1887) said f. inst. that

A, which is a disc with its highest refractive index centrally, may behave as a cylindrical lens thus producing the varying optical images. However, this clearing up of the central part of A may also be seen in fixed, non-stained muscle preparations cut in sections of a thickness of $5\ \mu$ (the author's observation), and by means of the electron microscope *Hall et al.* (1946) have found Q_H in fixed myofibril preparations. This indicates that there is an actual variation of the structure in the A-band. In accordance with this *W. J. Schmidt* (1937, p. 184) in some cases found a decrease of the birefringence in the central part of A. *Jordan's* explanation (1934) that Q_H indicates a preliminary contraction stage cannot be acknowledged; in living muscle fibres of mammals this clearing up is not observed especially in connection with contraction, neither is it observed in the experiments in which the variation of the cross striation during single contraction is recorded in relation to time. On the other hand Q_H is often seen in resting fibres, both in fibres at equilibrium length (fig. 3a) and in extended fibres; it appears most clearly in extended fibres.

M.

Stübel (1920) distinguished between M_x and M. M_x was only observed in living fibres, was often asymmetrically located in A, and was considered an optical phenomenon in contrast with the actual M which was preferably found in fixed preparations and always situated in the middle of A. According to *Heidenhain* (1911, p. 617) and *Häggquist* (1920, a—c) M has the same structure as Z, an assumption which has also been confirmed by electron microscopy. (*Hall et al.*, 1946).

In the A-segments of mammalian muscle fibres M-cross stripes are often observed (fig. 3b), but as they are not always situated in the middle of A they are probably false M-stripes, i. e. *Stübel's* M_x caused by projection of Z from parallel displaced bundles of fibrils, or it may be due to the refraction in case of oblique orientation of the transverse discs in relation to the optical axis of the microscope.

N.

N-cross striation in the isotropic segments has been described i. a. by *Engelmann* (1873) who considers these stripes to be birefringent. According to the opinion of several authors

N is due to a series of interfibrillar granules (*Flögel*, 1872; *Retzius*, 1890; *Holmgren*, 1907) while others, e. g. *Jordan* (1934) and *Hall et al.* (1946) assume that N is conditioned by intrafibrillar refractive phenomena.

N has never been observed in the living mammalian muscle fibres examined by the author.

Helicoidal structure.

On account of certain appearances of the cross striation frequently found in muscle fibres, especially in fixed preparations, several authors have adopted the theory that there are spiral-shaped — helicoidal — structural elements in the muscle fibres (*Münch*, 1903; *Heidenhain*, 1911—1919; *Tiegs*, 1922, 1923, 1924; *d'Ancona*, 1929—1930; *W. J. Schmidt*, 1937, p. 217—218; *Aurell & Wohlfart*, 1936, 1938, and others).

It is in the first place *Heidenhain* (1911—1919) who has described the helicoidal structure in detail. In longitudinal sections of a muscle fibre it may be seen that the cross striae of two parallel bundles of fibrils are displaced in relation to each other in such a way that n muscle compartments in one bundle correspond to $n + 1$ compartments in the other — as in a Vernier scale. *Heidenhain* introduced the designation “nonius periods” or “Nonius Felder” for such structures, and he called them sphenoids when they appeared as wedge-shaped, interposed, surplus compartments. *Aurell & Wohlfart* (1936, 1938) considered both the Z-membranes and the anisotropic fibril segments of skeletal and cardiac muscles to be arranged in the shape of a “winding staircase” (helix) all cross striae forming a continuous band in the longitudinal direction of the fibre.

Numerous investigators deny the existence of an actual helicoidal structure in the muscle fibres, maintaining that the above mentioned misleading appearance of the cross striation has merely been caused by a relative displacement of the myofibril bundles, namely: *Diamara* (1931), *Marcus* (1932), *Bruno* (1932), *Jordan* (1933) and *Speidel* (1937—1938). *Feneis* (1939) has demonstrated that by turning the isolated muscle fibre a few degrees about its longitudinal axis it is possible to make the nonius-like images disappear. The two halves of a nonius period are in different planes, and the image appears

when parts of the cross striation which are situated beneath each other are projected upon the same plane.

According to *Buchthal & Knappeis* (1940) the appearance of the diffraction spectra of living muscle fibres is not consistent with the existence of helicoidal structural elements in the fibres. A helicoidal structure might be considered to be composed of a system of optical gratings with different axes and would thus give a superposed diffraction pattern. By stretching muscle fibres considerably *Buchthal & Knappeis* have been able to observe diffraction spectra which were inclined in relation to the horizontal axis, an observation which indicates a displacement of the fibrils, but they did not observe any actual superposed diffraction patterns. Under most conditions the muscle fibres exhibited simple diffraction spectra which is a clear indication that the cross striation of the fibres is real.

It might have been assumed that this investigation had put an end to the theory of a helicoidal structure, but in a monograph on the intercalated discs of the cardiac muscles *Aurell* again takes up the helicoidal structure for discussion (*Aurell*, 1945, pp. 32—55), maintains his previous assertion and demonstrates the various images of the helicoidal structures by means of carrot models, while he criticizes *Buchthal's & Knappeis's* work (1940) rather sharply; but obviously *Aurell* has not made himself sufficiently familiar with these investigations. *Buchthal & Knappeis* found that normally the fibres exhibit a simple diffraction spectrum which is incompatible with a helicoidal structure of the fibres, it cannot thus be a fact — as *Aurell* maintains — that all muscles have a helicoidal structure. Only by maximum stretching of individual fibres was it possible to produce atypical diffraction patterns which might be due to displacement of fibrils. *Buchthal & Knappeis* ought perhaps to have emphasized that such fibres at equilibrium length or at moderate elongation had shown simple diffraction spectra. *Aurell* states that only the microscopical section, i. e. fixed preparation, is able to yield information as to the helicoidal structure, but by fixing displacement of fibrils is very easily caused.

There is consequently no reason to discard the theory that displacement of the fibrils is the cause of the helicoidal-like microscopical images, while nonius periods in unstained pre-

parations may also be caused by bundles of fibrils in different planes being projected on to the same plane.

These findings have been confirmed by experiments on living mammalian muscle fibres as the nonius periods and, in a very few cases, sphenoids observed have in particular been found in highly extended fibres, and by observing the muscle fibres while gradually being extended it has in some cases been possible to follow the displacement of the fibrils and the development of the nonius periods.

The Myofibrils.

It has for a long time been realised that the muscle fibres contain longitudinal structural elements and that these are to be found not only in A, but also in I — indeed, in the form of myofibrils they extend continuously throughout the length of the muscle fibre.

In living, unstained muscle fibres the myofibrillar structure cannot be seen clearly, an experience which was also confirmed by the study of mammalian muscle fibres. In case of very powerful magnification a slightly granular or indented zone becomes visible between A and I, and by stretching the fibres this zone is rendered more visible, a fact which indicates that the fibrils or bundles of fibrils are displaced slightly in relation to each other. *Speidel* (1937—38) found it likewise impossible under normal conditions to recognize the myofibrils of resting muscle fibres of the tadpole in situ, only in infrequent cases could he see: "delicate lines of tension". During extension and contraction of the fibres the longitudinal structure, however, appeared.

With dark field illumination the continuous fibril-structure in living muscle fibres is clearly seen (*Ettisch*, 1933). It will be mentioned in chapter IV that, during the study of the birefringence of muscle fibres, observation was made of the finely serrated contour of the arch-shaped deviation caused by muscle fibres to the interference line of the Babinet compensator. This serrated contour is a proof that the living muscle fibres contain longitudinal structural elements.

The fibrillar structure becomes more apparent when the osmotic conditions of the muscle fibres are altered (*Stübel*, 1920; *Zeiger & Schreiber*, 1927; *Boerner-Patzelt*, 1929), and or-

dinary fixing has the same effect (*Brücke*, 1858; *Hürthle*, 1909; *Heidermanns*, 1935). *Hürthle*, (1930—1931, a—c) examined the fibrillar structure by freezing the fibres as ice crystals then appeared in the space between the fibrils. It is also possible to split the muscle fibres longitudinally into fibrils or bundles of fibrils by mechanical means (*Ernst & Kellner*, 1936, a—d) — muscle fibres of certain animals are actually apt to disintegrate into fibrils spontaneously (*Heidenhain*, 1911, p. 578). After boiling the muscle fibres it is particularly easy to split them into bundles of fibrils.

With regard to the magnitude of the fibrils there is much disagreement. Some investigators find the diameter of fibrils to be from $0.2\ \mu$ to $2\ \mu$, while others consider the myofibrils to be ultramicroscopical and maintain that the measured diameters are those of fibrillar bundles. It is not very easy to refute this assertion as it is a matter of definition what is meant by a myofibril. *Hürthle* (1931, b) states in his table of the minute structure that the microscopical fibril is composed of several elementary fibrils, and *Köllicker* (1866) found that the fibrils are often accumulated in "columnae", but according to *Heidenhain* (1911, p. 581) the bundles cannot either be considered structural elements of constant magnitude. *Buchthal*, *Knappeis & Lindhard* (1936) measured bundles of fibrils, the diameters of which were $6\text{--}8\ \mu$, in living muscle fibres of frogs and were of the opinion that these bundles corresponded to *Cohnheim's* areas. They were also able to measure fibrils the diameters of which were $1.5\text{--}2\ \mu$. Such uniformity of the myofibrillar bundles have never been observed microscopically in the material of living mammalian muscle fibres examined by the author. Electron microscopy of fixed bundles of myofibrils has shown that the latter are composed of longitudinal myosin filaments with a diameter of $0.005\text{--}0.025\ \mu$ (*Hall et al.*, 1946).

It is probable that the thickness of the myofibrils is the same throughout their length (*Häggquist*, 1931, p. 129; *Schmidt*, 1937, p. 176). This means that in fusiform or tapering muscle fibres not all of the myofibrils extend from one end of the muscle to the other, a fact which is of importance for the understanding of the transmission of tension from the myofibrils to the connective tissue of the muscle.

When studying the arrangement of the myofibrils in the muscle fibres it is necessary to use fixed preparations. *Cohn-*

heim (1865) described the appearance of the fibre cross section found in frozen muscle fibres as "divided in areas" ("feldförmig"). According to Kölliker (1866) this "Säulchenfelderung" shows the cross sections of the myofibrils separated by sarcoplasm. Schaffer (1893) demonstrated that in some muscle fibres the myofibrils are more uniformly distributed throughout the cross section of the fibre — "Fibrillenfelderung" — and that in longitudinal sections such fibres appear clear or bright ("helle") in contrast with the dim ("trübe") muscle fibres, the cross section of which exhibit "Säulchenfelderung". As Knoll (1890, 1891) he was of the opinion that the latter muscle fibres contain more sarcoplasm than the clear muscle fibres. On the other hand he contested the assertion put forward by Knoll (1891) that Cohnheim's areas should be produced by fixing, even if he found that fixatives could alter the appearance of the cross section somewhat. Some investigators still hold that the different appearances of the cross sections are indicative of different properties of the muscle fibres (Danziger, 1937). (conf. p. 30). Others consider the cross sectional images to indicate different functional conditions of the fibres (Knoll & Barkley, 1940, conf. Hürthle's "Fibrilbündelung" during contraction, 1931, c). Muscles from various classes of animals, however, exhibit difference in the arrangement of the fibrils, e. g. the myofibrils of muscle fibres from fish are arranged in concentric rings (Görss, 1939).

The segmenting of the fibrils has already been mentioned and their minute structure will be discussed in a later section.

Spirally wound myofibrils.

Spirally wound myofibrils (Bataillon, 1891) are described as bundles of myofibrils, 2—24 μ thick, (Wohlfart, 1932; Voss, 1932), which winds in a spiral round the axis of the fibre, most frequently outside the other fibrils below the sarcolemma. The inclination of the spirals is very low, for which reason they appear as "Ringbinden" (Heidenhain, 1918, a and b) in cross sections of the fibres. They are usually only found in a part of the total length of the fibre as longitudinal fibril bundles bend and proceed along a spiral path. Transitional forms in which the myofibrils form irregular networks have also been described (Thulin, 1914; Bucciantie & Luria, 1937).

Voss (1932, 1935) advanced the hypothesis that the spiral

myofibrils were artificial products caused by the fixing, but *Bucciante & Luria* (1937) assert that they have observed winding fibrils in non-fixed and unstained fibres. *Bergstrand* (1938) has described the spiral myofibrils in details, and *Wohlfart* (1932—1938) has also made a rather thorough study of them. *Wohlfart* does not think that they indicate pathological processes, as described by previous investigators, i. a. *Heidenhain* (1918, a and b), *Slauck* (1921), *Schütz* (1922) and *v. Zalka* (1933). On the other hand *Wohlfart* is of the opinion that they have an unknown, specific function.

It has not been possible to ascertain the existence of myofibrils of spiral or otherwise irregular course in the isolated muscle fibres from the mammalian muscles examined in the present investigation. Unfortunately it was technically impossible to isolate intact muscle fibres from the eye muscles where spiral fibrils are supposed to be particularly frequent (conf. *Thulin*, 1908, 1914; *Wohlfart*, 1932, b).

The Sarcoplasm.

According to *Pischinger* (1931) sarcoplasm is a peculiar solution of numerous, to a certain extent unknown, substances, in which physico-chemical disturbances will very easily cause the precipitation of certain components; only I-granules may occur in vivo (*Pischinger*, 1931, conf. also *W. J. Schmidt*, 1937, p. 191). *Speidel* (1937—1938) found fine granules at the boundary of the A-discs in the tail muscles of tadpoles in vivo — a pair at each myofibril. *Holmgren* (1907, 1913), *Thulin* (1909) and others have ascribed great importance to the sarcosomes, distinguishing between A- and I-granules according to the location, and have described changes in morphology and location during contraction, but, as already mentioned, on fixed material. *Marcus* (1920—1922) considered deposits on the myofibrils to be the cause of the A- and I-cross striation, and according to *d'Ancona* (1930) the isotropic cross striae are due to isotropic I-granules, while *Liang* (1936) was of the opinion that the I-cross striation is produced by a netlike, isotropic structure. Only external influences will cause the latter to disintegrate into sarcosomes.

In living mammalian muscle fibres it has not been possible to distinguish between the sarcoplasm and the more solid

elements of the fibres, and definite interfibrillar granules have not been recognizable.

The hypothesis that the sarcoplasm is contractile, which has been propounded by certain investigators, will be discussed in chapter III.

By means of special staining methods *Arnold* (1909), *Studnitz* (1935, b) and others have tried to investigate the location of glycogen in the muscle fibres. *Studnitz* found that glycogen in resting muscles is found both in A and I, mostly in A. The stronger and the more lasting the irritation of the muscle the more the glycogen will accumulate in A. During the restitution period the newly formed glycogen appears mostly in I. Against these experiments, which were carried out on fixed material, the objection has been raised that no allowance was made for the rapidly occurring glycolysis (*Buchthal, Knappeis & Lindhard*, 1936). *Studnitz* does not make any allowance either for the transport of liquid (sarcoplasm?) from A to I during the contraction.

Fatty granules have in particular been observed in embryonic muscle cells (*Borghese*, 1937) and in connection with certain pathological conditions (*Slauck*, 1930, 1932, 1936, pp. 412—431, *Basile*, 1939).

By means of the specific absorption of ultraviolet light of the adenine derivatives *Caspersson & Thorell* (1942) have shown that in living, resting fibres the major part of adenine derivatives are to be found in the I-segments, in exhausted fibres they are also found in the A-segments.

Red and white muscle fibres.

The fact that some muscles are red and some white is not due to a different content of blood, but to a varying content of a certain pigment dissolved in the muscle plasm (*Kühne*, 1865). According to *Ranvier* (1874, a) the red muscles have a greater latency and their contraction is more slow and more lasting than that of white muscles. In all muscles of vertebrates there are, however, both pale, homogeneous ("helle") muscle fibres and darker, granulated ("trübe") fibres with longitudinal striation, but there are some muscles in which either one or the other type of fibre is completely dominating (*Grützner*, 1883). *Needham* (1926) has given a summary of these problems and *Denny-Brown* (1929) has shown that there is

consistency between the histological appearance and the mechanical reaction of the muscles, while *Danziger* (1937) found a relation between the function of the muscle and the occurrence of "Fibrillen-" or "Säulchenfelderung", but not between the colour of the muscle and these structural images.

It is still an open question, whether it is justified histologically to distinguish so sharply between the two kinds of fibres, and whether the mechanical functions of the two kinds differ. The marked divergence of the observations seems to indicate that the final answer will be in the negative.

In the examined mammalian muscles no differences between the fibres have been observed, neither with regard to colour and brightness nor with regard to mechanical properties. In *Buchthal's* material of muscle fibres from frogs such differences have not been ascertained either.

The Muscle Nuclei.

The muscle nuclei have especially been investigated by *Schiefferdecker* (1911—1919) who, in a very comprehensive work, has tried to establish a relationship between the function of the muscle and its number of nuclei, size of nuclei etc.

Calculation of the cross section of the nuclei and the number of nuclei per fibre cross section is, however, erroneous if no allowance is made for the degree of stretch of the muscle fibres. The elongation of the nuclei during stretch is not necessarily proportionate to that of the fibres, as the elasticity of muscle fibres and nuclei need not be identical. It has not been possible, by measuring the length of nuclei in fixed preparations, to establish any parallelism between height of compartments and length of nuclei. In cross sections relatively more nuclei will consequently be found in relaxed or contracted muscles than in stretched muscles.

The length of the nuclei varies from 5 μ to 12—15 μ and their thickness may be as great as 5—6 μ , they are described as fusiform or lentiform, ellipsoidal or rod-shaped and always with their longitudinal axis parallel to the longitudinal direction of the fibre. According to *Schiefferdecker* the location of the nuclei in the muscle fibres differs in different animals and in different muscles in the same animal. In vertebrates and birds the arrangement of the nuclei in the fibres is prefer-

ably peripheral, below the sarcolemma, while in fishes and amphibians the nuclei are more uniformly distributed over the cross section of the muscle fibres.

It is not possible to recognize the nuclei in unstained preparations with ordinary illumination, as their refractive index is not sufficiently different from that of the other components of the fibre. Only in a few cases will it be possible with a reduced illumination to distinguish them as turbid spots in the muscle fibre.

The Sarcolemma.

The sarcolemma does not only function as a cell membrane, but it is also supposed to transmit tension from the contractile substance to the connective tissue of the muscle. As moreover the sarcolemma must possess a considerable ability to alter its shape, because it must be able to follow the varying length of the contractile substance, it is obvious that great demands are made upon its structure.

Already *Pappenheimer* (1908) has shown that sarcolemma consists of a homogeneous basic substance and a network of collagenous fibrils in definite patterns. *Nagel* (1935) has especially studied the mechanical properties of the sarcolemma and emphasizes its elasticity; by a strong shortening of the muscle fibre the surface of the muscle cylinder becomes smaller without the sarcolemma becoming slack or wrinkled. Sarcolemma may be stretched to four times its original length and the alteration will remain reversible. *Ramsey & Street* (1940) have asserted that the length-tension diagrams of the resting muscle fibres are exclusively conditioned by the sarcolemma; they found similar length-tension curves for intact muscle fibres and muscle fibres with damaged interior, but intact sarcolemma. *Sichel* (1941) has shown that these observations cannot be correct. In chapter V we shall discuss this problem further.

It is not fully elucidated whether the elasticity of the sarcolemma is exclusively due to the texture of the network in connection with the basic substance or whether the fibrils in sarcolemma are elastic. *Bairati* (1938) contests the latter, he is of the opinion that the fibrils in sarcolemma are all collagenous; they are all birefringent. At the ends of the

muscle fibres sarcolemma may behave differently. According to *Lubosch* (1937) it may either be completely lacking here or it may continue in the shape of a sheath enveloping the continuation of the myofibrils, or it may form a fine-mesh network covering the ends of the muscle fibres and forming part of the tendinous fibrils which emerge from the muscle fibre.

It is not possible to observe the sarcolemma by microscopical examination of living, unstained muscle fibres under normal conditions. In longitudinally compressed fibres it is possible to observe a wrinkling of the surface of the fibres. An actual festooning with retraction of sarcolemma corresponding to the Z-membranes has not been seen, however. When the muscle fibres are injured the sarcolemma is often observed to remain intact while the content of the fibre is retracted.

The Connective Tissue of the Muscle.

Schiefferdecker (1911—1919) has described the nature of the connective tissue of various muscles, but has taken a special interest in the occurrence of elastic connective tissue. *Feneis* (1935) on the other hand examined the connective tissue in relation to the function of the muscle and pointed out that the connective tissue must permit high mobility between muscle fibres and fascicles, as the muscle fibres to a certain extent work independently of each other. In order to reduce the internal resistance of the muscle membranes of connective tissue, so-called displacement membranes, are inserted between the fascicles and connected to the latter by means of "neutral connecting threads", which run parallel to the tendons and convey vessels and nerves to the muscle fibres. The displacement membranes often contain adipose tissue, and their elasticity and ability to be displaced are obtained by a specific arrangement of inelastic material. According to *Nagel's* investigations (1935) the collagenous fibril bundles follow a course from the neutral connecting threads in an oblique direction round the muscle fibres, in the shape of "adventitious bundles", and after having reached the individual fibres they wind in spirals round the latter and finally they ramify and enter into connection with the "tricot-like" network of endomysium and sarcolemma. The distance be-

tween the windings of the spiral depends on the degree of stretch of the muscle. In highly stretched muscles the adventitious fibril bundles are almost parallel to the muscle fibres, while they are at right angles to the axis of the muscle fibres and sometimes also wrinkled when the muscle fibres are shortened during contraction. On account of this arrangement the connective tissue of the muscles permits relatively free displacement of the muscle fibres and acts merely as an increasing elastic resistance at high degrees of stretch.

On account of the above mentioned, the viscous-elastic properties of the muscle as a whole will be different from those of the isolated fibres; in the muscle it will be quite justifiable to speak of an internal frictional resistance — a true viscosity — while the viscous property of the individual muscle fibre is due to other causes which will be discussed in chapter V.

It has been shown on muscles of the frog that anisotropic segments of any fibre nearly always adjoin the anisotropic segments of adjacent fibres (*Buchthal & Knappeis*, 1940). *Buchthal & Knappeis* were guided towards this observation by their investigations on the diffraction spectra of muscle fibres, the muscle fibre bundles exhibiting sharp spectra exactly as isolated fibres, a fact which indicates that the gratings in the individual fibres are parallel. When the resting muscle is not subjected to any external mechanical influence the connective tissue thus maintains the position of the muscle fibres fairly accurately.

In mammalian muscle fibres it has likewise been possible to observe the cross striae to be at the same level in adjacent fibres at equilibrium length at rest (fig. 1 b). Both stretch and contraction result in relative displacement of the fibres, the displacement being usually reversible.

Numerous attempts have been made to elucidate the process according to which tension is transmitted from the contractile substance to the connective tissue by examining the behaviour of myofibrils, sarcolemma and connective tissue at the ends of the muscle fibres.

Ranvier (1889, p. 393) stated that there is a cementing substance between muscle fibres and tendons — the so-called apposition theory. Against this it was emphasized, i. a. by *Schulze* (1912), that the myofibrils are continued directly in tendinous fibrils, and that tension is transmitted directly

through these elements. Later on many investigators have accepted *Schulze's* theory and confirmed the continuity between tendinous fibrils and myofibrils, e. g. *Butcher* (1933), *Schüle* (1935), *Studnička* (1937) and *Speidel* (1937—1938). According to *Wöhlisch* (1932) the micellar structure of the tendinous fibrils and myofibrils is the same, and by polarization-optical investigations *W. J. Schmidt* (1936) has shown that the direct continuity between the tendinous fibrils and myofibrils is found everywhere in vertebrates, but that there is a sharp boundary between the myosin of the myofibrils and the collagen of the tendinous fibrils.

Häggquist (1920, 1926, 1931, p. 223—233) on the other hand claims that muscular tension is transmitted through the Z-membranes to the sarcolemma and to the endomysium. In support of this theory he stated i. a. that when a muscle fibre is fusiform, all of the myofibrils of the fibre cannot extend from one end of the fibre to the other. The absolute length of the myofibrils therefore differs, and their absolute shortening during contraction consequently also differs. This would result in a highly uneven distribution of tension, if the force from the individual myofibril was transmitted directly to the tendinous fibrils. Transmission of tension to the network structure of sarcolemma and endomysium can however explain the mechanical phenomena much better. *Häggquist* also supported his theory by histological observations of the transition between muscle and tendon. As *Péterfi* (1913) he found that here the direction of the fibrils was at right angles to the acting forces, and he could not find any direct continuity between tendinous fibrils and myofibrils, while the fibrillar structure of sarcolemma and endomysium was continued in the tendinous tissue. *Häggquist's* theory was accepted i. a. by *Rydén & Wohlfart* (1932), by *Lindhard* (1926) and by *Nagel* (1935). The latter observed a wrinkling of the adventitious collagenous bundles in contracted fibres, a fact which, according to his opinion, indicated that the tension during contraction must be transmitted along the whole surface of the muscle fibre.

Others, as e. g. *Sobotta* (1924) and *Clara* (1931) suppose that the contractile force is transmitted in both ways. *Lubosch* (1937) has given a good review on these problems. He assumes a direct transmission of force to be essential, but agrees that a great part of the force may be transmitted through the peri-

mysium. Histologically he has found cases in which there is continuity between tendinous fibrils and myofibrils without an intervening sarcolemma. Cases in which there is connection between tendinous fibrils and myofibrils through the fine-mesh sarcolemma network. And cases in which sarcolemma forms a boundary layer in the shape of a 3-dimensional fibrillar network ("Faserfilz"), externally connected to tendinous fibrils, internally to light, non-cross striated threadlike structural elements which continue to the myofibrils. — And finally cases in which the cross striation continues right down to the sarcolemma so that the myofibrils are attached to the fibrillar network and possibly also have individual independent continuations.

The Motor Innervation of the Muscle.

Eccles & Sherrington (1930) found 200—650 motor units per muscle in various of the muscles of the extremities of the cat. *Clark* (1931) found, by counting nerve and muscle fibres, that the motor unit of the muscles of the extremities of the cat includes 120—165 fibres. *Cooper's* experiments (1929) indicated that the arrangement of the motor nerves to the m. sartorius and m. tenuissimus of the cat is such that the muscle fibres which function simultaneously in case of partial contraction are arranged in series extending through the whole length of the muscle. If this was not the case the active muscle fibres would merely stretch the inactive ones, and the efficiency would be reduced.

Pathologico-anatomical investigations e. g. by *Slauck* (1923, 1930, 1932) and by *Wohlfahrt & Wohlfahrt* (1935, pp. 94—97) have shown that muscle fibres innervated by the same anterior horn cell is collected in small bundles of 10—20 fibres. One motor unit comprises several small bundles and it is reasonable to assume that in muscles in which individual fibres do not extend from tendon to tendon small bundles belonging to the same motor unit are arranged in continuation of each other.

The motor end plates are often situated in certain zones of the muscle (*Zöbisch*, 1934; *Lezawa*, 1939, 1941).

It is generally assumed that there is only one motor nerve fibre for each muscle fibre, some authors have, however, found

two or more, (i. a. *Agduhr*, 1919; *Harreveld*, 1939; *Katz et al.*, 1941). *Agduhr* (1919, 1939) even speaks of a plurisegmentary innervation. *Häggquist* (1938—1940) has advanced the theory that there are two systems of nerve fibres, coarse nerve fibres, which transmit contraction, and fine nerve fibres, which transmit "tonus" and innervate special "tonus muscle fibres", but this theory has been questioned (*E. Krogh*, 1946; *Leksell*, 1945, p. 75). Several authors (i. a. *Boeke*, 1927; *Agduhr*, 1919; *Frank*, 1920) have observed small accessory end plates which were supposed to originate from the autonomic nervous system and to be of importance to muscle tone etc. (conf. i. a. *Kuré*, 1931), but these observations and theories have also been much disputed. When checking *Boeke's* investigations *Wilkinson* (1931, 1934) observed sympathetic nerve branches in relation to the blood vessels only.

A review of the structure of the motor end plates is given e. g. by *Stöhr* (1931), *Boeke* (1932) and *Bielschowsky* (1935). When the motor nerve branch reaches the muscle fibre it loses the myeline sheath; the outer sheath, neurilemma, joins the sarcolemma and the neurofibrils enter the end plate and split subsarcolemmally into a complex fibrillar network, the fibrils of which always terminate in closed loops. Around the neurofibrillar plexus and between the latter and sarcolemma the muscle sole or bed is situated; it contains more nuclei and is especially rich in granules, it forms a boundary layer towards the sarcoplasm. In mammals the end plate is usually fusiform, with its longest diameter in the direction of the longitudinal axis of the muscle fibre — it extends across several compartments, but is only of a small thickness, i. e. 1—10 μ .

The existence of a periterminal network, extending from the actual neurofibrillar network of the end plate through the sarcoplasm to the myofibrils (*Boeke*, 1909, 1926, 1932), is denied e. g. by *Péterfi* (1935), *Wilkinson* (1931, 1934) and by *Buchthal & Lindhard* (1939, p. 51). The latter found a potential difference between the end plate and the muscle compartments which is not in conformity with a direct continuity between neurofibrils and contractile substance. On the basis of different effects of acetylcholine and potassium *Buchthal & Lindhard* (1942) assumed the existence of two functional boundary faces at the transition from nerve to muscle, one

between the neurofibrillar network and the muscle sole — here the acetylcholine presumably acts — and the other between sole and sarcoplasm — and here potassium is supposed to act.

In living muscle fibres the motor end plates can be seen as blurred parts on the side or upper surface of the fibres, they are most easily found by following the thin nerve branches and appear most clearly when the fibres are examined with reduced illumination.

By means of a special micro-technique *Buchthal & Lindhard* (1937—1942) could — as mentioned — apply various solutions, e. g. acetylcholine solution, locally to the end plate of isolated, living muscle fibres from lizards. Similar experiments have been performed by *Buchthal, Lindhard & Høpncke* (unpublished experiments) on isolated mammalian muscle fibres from the cat, and, just as in lizard muscles, it was possible to produce contraction by local application of very small amounts of acetylcholine (dose: abt. 10^{-5} μ g.) or very small amounts of dissolved potassium (dose: abt. 10^{-4} μ g.).

C. Quantitative Determination of the Cross Striation and of its Variation during Contraction.

Engelmann (1873—1881) was the first to perform exact quantitative determinations of the height of the compartments of muscle fibres and of the various layers of the compartment. He employed fixed as well as freshly excised, living muscle fibres from various insects, amphibians and mammals, and he found by measurement a ratio of the A-layer to the I-layer of 1 to 1; in arthropods A was slightly higher than I, while the opposite was the case in vertebrates. The ratio of A:I was fairly independent of the degree of stretch of the fibres. The material used by *Engelmann* for studying the structural changes occurring during contraction was mostly fixed insect muscles, as he asserted that the contraction waves to be observed in preparations fixed by means of perosmic acid were identical with the spontaneous contraction waves in living muscle fibres. Consequently the results have to be taken with

reservation. He found that during contraction the height of I decreases relatively more than that of A, and the volume of A increases at the cost of I; at the same time a reversal of the refractive indices takes place, I becoming darker than A during maximum contraction. According to *Engelmann* this is caused by a transport of liquid from I to A.

Krause (1873) and *Rollett* (1885, 1891) denied the occurrence of such a reversal of the cross striae during contraction; but *Krause*, who especially examined muscles from frogs and mammals claimed that only the height of I decreases during contraction.

Hürthle (1909) emphasized the advantages of measurement of the object above "subjective" qualitative observation. By means of his cinematographic method he photographed surviving muscle fibres of *Hydrophilus* and recorded the spontaneous contraction waves occurring here. According to *Hürthle* the height of compartment in *Hydrophilus* is 4–8 μ and I amounts to only $\frac{1}{8}$ of the total height. During stretch of the muscle fibres the ratio A:I is not altered. During contraction the total height of the compartment is reduced by 50 per cent, A decreases 60 per cent in height, while I remains unchanged or even increases by up to 30 per cent. This means that contraction takes place in the birefringent layer. *Hürthle* could not accept *Engelmann's* contraction theory with "swelling" of the A-substance, and he was unable to observe the reversal of the light re.raction described by *Engelmann*. He assumed that the sarcoplasm moves from A to I and that the rod-shaped, birefringent segments of the fibrils become shorter and thicker during contraction, but maintain their volume.

In insect muscles and in frog muscles *Meigs* (1908–1914) found a similar ratio A:I, A amounting to $\frac{9}{10}$ of the compartment. A state of contraction was, according to his observations characterized by a festooning with retraction of sarcolemma at Z and at M.

In investigations dealing with frog muscles *Frank* (1928) showed that all fixatives produce contracture of the cross striated muscles, while freezing with subsequent fixing in Carnoy's liquid at a temperature of -6 to -8°C does not produce any shortening of the muscles and thus probably yield a correct picture of the structure at rest. For examination of the structure during contraction he used muscles fixed

— without freezing — during tetanic contraction. In frog muscle preparations made in this way he measured the height of compartment to be $2.2\ \mu$ and the ratio A/I*) at rest to be 1.0—0.83. If the resting muscle is stretched the elongation will particularly occur in I, while the height of the A-layer is increased at the cost of I during isometric, tetanic contraction. When the ratio A/I in a resting, stretched, fixed muscle is 0.5—0.67 it will in a tetanized, stretched, fixed muscle be 1.11—1.16. By isometric contraction the ratio A/I may be altered to 2.2.

Hürthle (1930) questioned *Frank's* observations; he showed that the frog muscle loses its tension and its degree of shortening by being immersed in the fixatives during tetanic contraction. The histological picture of muscles fixed in that way does consequently not correspond to the contraction existing in vivo. *Hürthle* (1931) stated that the muscle fibres are more easily fixed in a contracted condition by being immersed in liquids of low temperatures (solid CO_2 dissolved in ether (temp. -70°C) or liquefied air (temp. -180°C)). He exsiccated the frozen muscle fibres in vacuum and examined the fibres both stained after embedding in paraffin and unstained. He succeeded in fixing muscle fibres at rest by leaving the muscles before fixing for one hour in air, in which they lose their contractility. The freezing results in ice crystals forming between the fibrils splitting the fibrils into bundles, this phenomenon being most pronounced in contracted fibres, as an expression of the "bundling" or "formation of columns" of the fibrils during contraction. *Hürthle* found that the anisotropic rods became shorter and more spherical during isotonic contraction, and he speaks of "Perlenquerstreifung" in contrast to "Stäbchenquerstreifung" as existing in resting fibres. The ratio A:I did not change during isotonic contraction as both A- and I-layers decreased in height, the birefringent substance still amounting to 50 per cent of the total height of the compartment. The height of the compartment of resting frog muscle fibres was measured by him to be $2.2\text{--}2.7\ \mu$.

In an excellent paper (1932) *Holz* has photographed fibres from m. cutaneus pectoris of the frog in situ, and measured photometrically on the negative film the ratio of the height

*) To facilitate comparison *Frank's* figures have been converted from I/A ratios to A/I ratios.

of the isotropic to that of the anisotropic layer, but unfortunately he has not stated the absolute values. He found that the heights of A are approximately the same as those of I in resting muscle fibres, perhaps A is slightly higher than I. During isotonic tetanus A is shortened more than I.

As examples of the widely differing conceptions of the muscular structure formed by different investigators it may be mentioned that *Diamara* (1925) and later on *Bruno* (1930) concluded that the cross striation is the same in vertebrates and arthropods as regards stainability, but the polarization-optical properties differ, the light substance is isotropic in arthropods while in vertebrates it is anisotropic. During contraction the A-stripes in arthropods are displaced towards Z, forming contraction bands (= C), while in vertebrates the latter are formed by A which is drawn towards M. *Jordan* (1934) as well as *W. J. Schmidt* (1937, pp. 171—175) contested this conception. *Jordan* explained the fallacy by assuming that the examined muscle fibres from vertebrates had been slightly contracted, and *W. J. Schmidt* ascribed the mistake to defective focusing.

Jordan (1919, 1933, 1934), however, was also of the opinion that anisotropy and stainability were two different phenomena, not necessarily interdependent. In muscle fibres of wasps he found that the cross striation is reversed during contraction, a lighter zone appears in the middle of A, and the two halves of A are displaced towards Z, thus forming a contraction band possibly in conjunction with the accessory discs (N). The location of the anisotropy is not, however, altered.

W. J. Schmidt (1937, p. 171) likewise agreed with *Engelmann* as to the reversal of stainability and refraction of the anisotropic and isotropic substances during contraction. *Schmidt* did not ascribe this merely to intrafibrillar changes, but also to accumulation of I-granules against the Z-membrane.

Studnitz (1935, a, 1936) on the other hand found that in arthropod muscles A is always stained more intensely than I, whether the muscle is resting or contracted. He recommended chloral hydrate-glycerine as the most gentle fixative and found that the height of A as well as of I decreases during pronounced contraction (A 50—70 per cent and I 30—50 per cent); accordingly I should also be contractile; these findings are, however, not made under sufficiently well-defined experimental

conditions, (conf. *Buchthal, Knappeis & Lindhard, 1936*). By measuring various insect muscles *Hildégard Brenner (1939)*, one of Studnitz' pupils, claims interdependence between the ratio A:I, the height of the compartment and the speed of reaction of the muscle fibres, the more rapid the reaction of the muscle, the lower the height of the compartment and the smaller the difference between the height of A and I. This work will be discussed in chapter III (p. 126).

Speidel (1937—1939) has examined living muscle fibres of mammals, amphibians and arthropods; his results are mostly based upon investigations of muscles of the tail of the tadpole in situ. Here the height of the compartment amounts to 2.1μ and I is elongated more than A during stretch. During contractions when the fibres are shortened by up to 25 per cent I is shortened relatively more than A; in case of intense contractions (shortening above 45 per cent) contraction bands are formed with an accumulation of dark, birefringent substance at Z. In frog fibres *Speidel* could produce slowly travelling contraction waves just as they have been observed in insect muscles.

In 1936 *Buchthal, Knappeis & Lindhard* published their investigations on the structure of muscle tissue, carried out on isolated, living fibres. They measured the height of compartment and of the A- and the I-substance at different degrees of stretch, at rest as well as during contraction. The measurements were carried out on microphotographs and checked photometrically. The technique has later on been improved and the results confirmed by *Buchthal & Knappeis* on a more comprehensive material. (*Buchthal, 1942, pp. 48—64*). These investigations, which are discussed further in the following chapters, showed:

At equilibrium length the height of the compartment of muscle fibres from m. semitendinosus of the frog amounts to 2.2μ with a variation of about 10 per cent. The ratio A:I at equilibrium length is a more constant value, it varies 5 per cent. The A-substance amounts on an average to 61 per cent and the I-substance to 39 per cent of the total height of the compartment. When the fibres are stretched the elongation of the A-substance is slightly larger than that of the I-substance. At an extension of the fibres of 50—60 per cent A thus amounts to 65 per cent of the height of the compartment. During isometric contraction the A-substance is shortened

and the I-substance correspondingly elongated, the height of the two layers may be almost identical during contraction. Changes of the cross striation during tetanic contraction are however not equally pronounced in all preparations and are also to a minor extent dependent upon the degree of stretch of the fibres, the changes of ratio A : I during contraction being smaller for moderately stretched fibres (length 120—130) than for fibres at equilibrium length and for fibres at high degrees of stretch.

According to *Buchthal, Knappeis & Lindhard* an active shortening of the I-substance does never occur, not in case of tensionless contraction either. It is consequently the A-substance which alters its equilibrium length during contraction, but the I-substance also plays an active part in the contraction process as the stiffness of the I-substance is increased during contraction — as shown by *Buchthal* (1942, p. 57).

Knappeis, Lindhard & Topsøe-Jensen (1940) by means of the same experimental methods determined the height of the compartment in isolated, living muscle fibres from *Astacus fluviatilis* and *Carcinus maenas* to be 6.3 and 6 μ respectively at equilibrium length. In both cases A amounts to 56 per cent of the height of the compartment in resting fibres, it is elongated relatively more than the I-substance at stretch and is shortened to 52 per cent of the height of the compartment during isometric, tetanic contraction.

The cardiac muscles of the frog were examined by *Knappeis & Lundin* (*Lundin*, 1944, p. 47). Here A likewise amounts to 60 per cent of the height of the compartment, but in case of passive stretch the cardiac muscles of the frog, in contrast to skeletal muscles, exhibit less stiffness of I than of A, I being elongated relatively more than A. At length 170 of the fibres A thus amounts to 59 per cent of the height of the compartment. In case of isometric contraction A is shortened while I is elongated in the same way as in skeletal muscle fibres. By means of micro-cinematographic recording with a frequency of exposures of 100 per sec., each exposure lasting for 0.4 msec. *Buchthal & Knappeis* (1943, a and b) succeeded in observing the changes of the cross striation during different phases of single contractions. Measurements of these microphotographs showed that, just as tetanic contraction, isometric single contraction is accompanied by a shortening of the A-substance and an elongation of the I-substance. The change reaches its

The fact that the muscle fibres possess both crystalline birefringence and form birefringence has been shown i. a. by *Stübel* (1923). He demonstrated this phenomenon by embedding fixed muscle fibres in media of different refractive indices. The variation of the total birefringence with the refractive index of the medium employed for embedding appears from fig. 4. At the lowest point of each curve the refractive

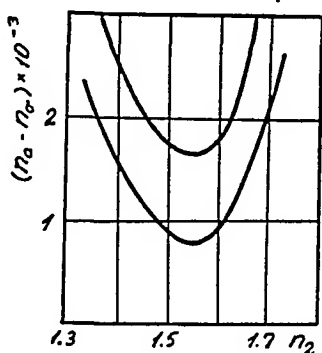


Fig. 4.

The dependence of the birefringence on the refractive index of the embedding medium (ac. to *Noll & Weber*, 1934). See text p. 44.

Preparation: Myosin thread (top curve).

Cross striated muscle fibre (bottom curve).

Abscissa: Refractive indices of the embedding media.

Ordinate: Birefringence measured with the preparations embedded in the various media.

index of the medium is equal to that of the micellae. In that case the form birefringence is zero and the total birefringence is solely determined by the crystalline birefringence of the micellae. *Stübel*, however, also found a negative birefringence in the muscle fibres caused by lipoid substances, but later investigators i. a. *Noll & Weber* (1934) have been unable to confirm this observation.

O. Wiener (1909) has elaborated the mathematical theory of the form birefringence, and *Ambrohn* (1916) has confirmed this theory. In 1929 *K. H. Meyer* has given an excellent summary of the structure and chemistry of the micellae and related problems, which are of special interest to biologists.

The interest in birefringence investigations of muscle tissue was renewed when in 1930 *Edsall* succeeded in preparing a pure myosin solution, and when, later on, i. a. *Boehm & Weber* (1932) were able to prepare myosin threads. *Noll & Weber* (1934) concluded that the minute structure of the myosin

threads is the same as that of the A-segments of the muscle fibres as great conformity exist especially with regard to crystalline and form birefringence. As *Stübel* they used the imbibition method, the technique, however, being improved according to *W. J. Schmidt* (1934, b). *E. Fischer* (1936, 1938, 1944) has likewise studied the birefringence of the muscle fibres and shown that conditions are practically the same in myosin, in smooth muscles and in the A-segments of the cross striated muscles. In the two latter the crystalline birefringence amounts to approximately 35 per cent and the form birefringence to 65 per cent of the total birefringence.

The change of the birefringence during muscle contraction.

According to *Brücke* (1858) the birefringence of frog muscles does not change during isometric contraction; the same observation was made by *Hermann* (1880) while *Valentin* (1880) found that the anisotropy decreased to zero during isotonic contraction. *V. Ebner* (1882, pp. 89—93) found that the anisotropy decreases temporarily during isotonic muscle contraction, while *Exner* (1887) did not observe any change. Several investigators, i. a. *d'Ancona* (1931) and *W. J. Schmidt* (1937, p. 205), have later on been able to confirm the "negative Schwankung der positiven Doppelbrechung" mentioned by *Valentin*. It is particularly *v. Murall's* work in this field (1932—1933) which deserves to be emphasized. By means of his experimental arrangement he could at the same time record the change of birefringence and the variation of tension during contraction photographically. In the frog muscles examined he found a decrease of the birefringence up to 40 per cent during isometric contraction with two minima, "Anspannungsschwankung" and "Erschlaffungsschwankung"; he supposed that it was the crystalline birefringence which changed. According to *E. Fischer* (1936, 1938, 1944) the change of the birefringence during contraction is dependant on the initial length of the muscle, and this fact is probably the explanation of the differing results obtained by earlier investigators. *Bozler & Cottrell* (1937) confirmed *v. Murall's* observations, but they also found a certain dependence on the initial tension; they suggested that the change of the birefringence during contraction was in the first place due to a change of the length of the muscle.

Buchthal & Knappeis (1938) emphasized the objections against the use of whole muscles for quantitative determinations of birefringence, as that — on account of the structure of the muscle — would mean experimental conditions which would vary far too much. They also emphasized the sources of error prevailing when working with fixed muscle fibres: shrinkage, alteration of the ratio $A:I$. They themselves only used living, isolated frog muscle fibres and, as *v. Muralt*, they observed the birefringence to decrease during contraction and to amount to 15—30 per cent. If the muscle is poisoned with monoiodoacetic acid the birefringence does not decrease during contraction. After a prolonged series of contractions the birefringence remains low for some time and at the same time the content of lactic acid in the muscle fibre is increased. If lactic acid is added to the Ringer solution surrounding the muscle fibre a decrease of birefringence will likewise occur. These investigators considered the change of the birefringence to indicate a combination of proteins of the muscle fibres with acid and not to be a specific effect of anions.

Roentgen-spectroscopical investigations.

In 1926 *Herzog & Jancke* by an X-ray-spectroscopical examination of dried frog muscles confirmed that the arrangement of the molecules of the micellae must be crystalliform and that this crystalline property increases on drying or on stretching of the muscles. Later, when the knowledge of X-ray-diffraction patterns had been increased and the technique improved (*Frey*, 1928; *Boehm*, 1931; *Astbury*, 1934; *Frey-Wyssling*, 1938) especially *Weber's* (1934, b) investigations on myosin threads further elucidated the minute structure of the myofibrils. *Weber* found that the individual micella consists of 10—20 "Hauptvalenzketten", the distance between the latter is about $1\text{ m}\mu$. The micella itself is about $1\text{ m}\mu$ thick, 50—100 $\text{m}\mu$ long, and the distance between the individual micellae is only a few $\text{m}\mu$. The correctness of *Weber's* calculations has been questioned by *F. O. Schmitt* (1944). By X-ray analysis *Astbury & Dickinson* ascertained consistency between the structure of the micellae in living muscles, in myosin threads and in keratin. When the muscle fibre has its normal length the protein molecules,

which are parallel to each other, are half folded; during stretch the molecules are straightened out, during contraction they become further folded, but their arrangement parallel to each other remains fairly unchanged.

Other optical properties of muscle tissue.

Thanks to the alternate anisotropic and isotropic cross striae the muscle fibre behaves as an optical grating, e. g. as a Rowland grating, and when monochromatic light of known wave length is used it is possible on the basis of the spectrum to calculate the grating constant, i. e. the height of compartment. *Ranvier* (1874, b) already knew the diffraction spectrum of the muscle, and later on it has been examined by *Bernstein* (1895), *Nicolai* (1936) and *Sandow* (1936). As already mentioned *Buchthal & Knappeis* (1940) showed — on the basis of the diffraction spectrum — that the cross striation of isolated, living frog fibres is not helicoidal. By analysing the caudiform expansion of the spectra, which is due to a diffraction phenomenon in the longitudinal structural elements of the fibres, *Buchthal & Knappeis* could show that the micellae are not arranged absolutely parallel to each other in resting muscle fibres at equilibrium length, but they become parallel both during stretch and during contraction.

Buchthal, Knappeis & Sjøstrand (1939) likewise examined the absorption and diffraction of the light by single muscle fibres. The light absorption of the muscle fibres is very low, it is not altered by stretching the fibres, but it is increased during contraction, a fact which is indicative of the different changes of the minute structure during extension and contraction respectively. The diffraction of the muscle fibre shows that the structural elements which cause the diffraction, i. e. the micellar bundles, have a diameter which is larger than the wave length of the light. Such investigations have also been performed on whole muscles, i. a. by *v. Baeyer & v. Muralt* (1935) and by *Schäfer & Göpfert* (1937), but here the experimental conditions become less exact on account of the complicated muscle structure.

The mechanical and thermo-elastic properties of the muscle fibres.

The thermo-elastic properties of muscle fibres indicate that the viscous-elastic system of the muscle fibre consists of flexible molecular chains, the orientation of which become absolutely uniform, "crystalline", when the fibres are stretched, while contraction results in folding of the chains and a subsequent development of a more amorphous structure. Investigations in this field which may be mentioned are i. a. works by *Wöhlisch* (1931, 1940, 1941) and by *Meyer & Picken* (1937).

The most important earlier investigations on the mechanical properties of muscle fibres performed on isolated fibres will be dealt with in chapter V together with the author's preliminary experiments on mammalian muscle fibres. For practical reasons the mechanical equivalent of the minute structure of the muscle fibres, which is based on the various investigations performed on living, isolated frog muscle fibres (*Buchthal, Lindhard et al.*, 1936—1946), will also be dealt with in chapter V. Here it shall only be mentioned that also as regards the viscous-elastic properties consistency has been found to exist between myosin threads and muscle fibres (*Weber*, 1934, a). According to *Dubuisson & Monnier* (1943) the length-tension diagrams of freshly made myosin threads, examined at a pH value of 7, are qualitatively very much like the length-tension diagrams of isolated frog muscle fibres found by *Buchthal* (1942), but — nota bene — of contracted fibres, not of resting fibres as should have been expected. *Singh* (1943), by examining myosin solutions, established the fact that if contraction is due to a physical change in the myosin molecules an alteration of the viscosity during contraction and a dependence on stretch must be expected; and this actually holds, especially for smooth muscles. As to this problem further reference should be made to summaries by *Dubuisson* (1939), *Ramsey* (1944) and *Fenn* (1945).

All the above mentioned methods of examination thus shows that there is consistency between the minute structure of myosin and muscle. Polarization-optical and X-ray-spectroscopical examinations both establish the high degree of orientation of the minute structure of the anisotropic segments of cross striated muscle fibres which is a prerequisite of con-

tractility. *Bailey* (1937) has shown that myosin is found in all the muscles examined by him and that its composition is remarkably uniform, even when taken from different species of animals. Thus there is experimental evidence that the contractile substance of the muscles is closely related to the myosin protein group.

Correlation between the chemistry and physics of muscle contraction.

The research work from the latest years has brought the solution of the greatest problem in muscle physiology a step forward — i. e. the problem of the conversion of the chemical energy of the muscle into mechanical energy during contraction.

Among the series of chemical actions which occur in connection with muscle contraction the break-down of adenosine triphosphate (= ATP) is the one which sets in first (conf. i. a. *Lundsgaard*, 1938). *Engelhardt & Ljubimowa* found (1939) that myosin is an essential link in the ATP break-down, it acts as ATP phosphatase. *Needham et al.* (1941) have shown that if ATP is added to a myosin solution it will result in reversible changes in the viscosity and flow birefringence, and according to *v. Szent-Györgyi* (1945) this should be due to alterations in the degree of aggregation and hydration of the myosin particles.

The muscle fibres contain about 0.025 per cent ATP, and according to *Caspersson & Thorell* (1942) the adenine derivatives have a specific power of absorbing ultraviolet light and their location in the muscle fibres have been determined by means of a special technique according to which absorption spectra of small objects can be measured.

Buchthal, Deutsch & Knappeis (1944, 1946) examined the effect of ATP and closely related compounds on isolated frog muscle fibres. The cell membrane of the muscle fibre is permeable to ATP. Addition of ATP to the Ringer solution — even in very small doses — will initiate a tetanic contraction of the muscle fibres. The birefringence decreases, but the change in the birefringence does not reach maximum until all the mechanical changes during the contraction are completed and the ratio A : I has again returned to its resting value. Tetanic contraction caused by electric stimulation is

accompanied by an immediate decrease of the birefringence of 20—30 per cent, but also in this case the birefringence does not return to normal value until the contraction has long ago ceased. Thus, the slow changes in the birefringence are not directly correlated to the actual contraction phase, but are considered an expression of the restitutional processes in the muscle fibre. In conformity with this a muscle poisoned with monoiodoacetic acid does not exhibit any slow change in the birefringence during ATP contraction — or during contraction produced by electrical stimulation. On the other hand changes in the birefringence will occur in spite of lacking mechanical contraction tension when ATP is applied to fibres which do not react on electrical stimulation. The actual contraction and the change in the birefringence are thus two functions, partly independent of each other, which seem to be caused by different structural changes in the protein molecules.

The break-down of ATP is the first of the known energy-producing reactions to set in. Previously all these reactions have been taken to belong to the restitutional phase, the investigations by *Buchthal, Deutsch & Knappeis* indicate that ATP, besides starting the restitution processes and causing a change in the birefringence, is also the factor which releases the actual contraction process. In the resting muscle ATP is either separated from the contractile substance (the myosin) or it is chemically linked so as to be inactive. The nerve impulse or the release of acetylcholine effects the contact between ATP and the contractile substance. Also in mammalian muscles *in situ* *Buchthal and Kahlson* (1944) could ascertain the contraction-producing effect of ATP — by close arterial injection.

E. Comparison between Living and Fixed Muscle Preparations.

As pointed out by *Hürthle* (1925, p. 109) there is no other animal tissue than muscle tissue in which such great differences between living and fixed material may be observed. In the following this fact is to be elucidated on the basis of available literature and of own investigations; and the significance which should be attached to investigations performed on fixed

material will finally be discussed — with regard to examinations of the normal structure and with regard to pathologico-anatomical investigations.

The vulnerability and lability of muscle tissue are i. a. due to a high water content (approximately 80 per cent). Drying of the muscles will result in severe shrinkage which — as shown by *Rubner* (1922) — is much more pronounced in the cross diameter of the fibres than in their longitudinal direction, when drying in air 76 per cent and 3.6 per cent respectively. The relative height of A and I is not altered essentially by drying (*Hürthle*, 1931, b). In arthropod muscles *Studnitz* (1935, a), however, found that by alcohol fixing the height of A decreases more than the height of I.

When muscles fibres are immersed in a hypotonic solution of neutral salt their structure will be altered — from normal cross striation, via *Stübel's* "simple cross striation" (1920) to granular disintegration — according to the hypotonicity of the solution. In pure water the cross section of a muscle fibre will increase considerably, at the same time the fibre will become shorter and the structure will gradually be completely destroyed (*Meigs*, 1937, p. 1105). Towards hypertonic salt solutions muscle fibres are more resistant. Such solutions produce a marked longitudinal striation and in some cases result in "contraction nodes" (*Stübel*, 1920; *Zeiger & Schreiber*, 1927; *Heidermanns*, 1935 and *Liang*, 1936). *Zeiger & Schreiber* (1927) were able to establish a rather comprehensive parallelism between the change caused by the ions in the muscle structure and their influence on muscle irritability.

Increased acidity will likewise change the structure of muscles — produce contractures — "Säurestarre" (*Ernst & Kellner*, 1936, c). Acids influence the birefringence (*Buchthal & Knappeis*, 1938), and an alteration of the pH level of the muscle fibres results in an appearance of sarcosomes (*Boerner-Patzelt*, 1929).

The fixatives used for histological purposes will usually cause shrinking of the tissue, although some of them will produce swelling. Often the fixatives will alter the acidity of the tissue, not only because of the actual pH value of the fixatives, but also because they may combine chemically with the tissue. This involves an alteration of the colloido-osmotic conditions during the fixing, so that e. g. precipitations may occur in the sarcoplasm (*Pischinger*, 1929, 1931). For the same reason

the affinity of the individual structural elements to stains will never be the same in preparations in vivo and in fixed preparations (conf. i. a. *Zeiger*, 1930). The fixatives often have a directly precipitating influence on proteins besides having other specific physico-chemical effects the nature of which it will be outside the scope of this work to mention (see *Zeiger*, 1938).

As shown, i. a. by *Hürthle* (1909—1930) and *Frank* (1928) it is impossible to fix living muscle fibres in their momentary functional condition by means of chemical fixatives. The assertion made by *Studnitz* (1935, a, 1936) that chloral hydrate-glycerine (6 parts of a 10 per cent aqueous chloral hydrate-solution + 1 part of glycerine) can fix muscles without producing artificial alterations of the cross striation, has been disproved by *Buchthal*, *Knappeis* & *Lindhard* (1936) and *Langer* (1937). The measurements which *Studnitz*' pupil *H. Brenner* (1939) has advanced to prove the reliability of this fixing method cannot be accepted either, as they exhibit great dispersion.

Hürthle (1931, a, c) on the other hand stated that by freezing it was possible to fix muscle fibres in a state of rest as well as in a state of contraction as mentioned on p. 39. But even in preparations treated in this way he could not observe uniform cross striation. If the muscle fibres are cooled gradually, the structure will be destroyed (*Fishback* & *Fishback*, 1932; *Ernst* & *Kellner*, 1936, a; *Speidel*, 1937—1939).

By applying various of the usual fixatives directly to living mammalian muscle fibres the author has been able to demonstrate the alterations produced by the fixing (table 1 and fig. 5). As soon as the fixative comes into contact with the muscle fibres it causes a violent shortening of the fibres. During the shortening it is impossible to see the cross striation, and a wrinkling of sarcolemma may often be observed. The fibres relax slowly, but it may last up to $\frac{1}{2}$ minute until the changes in the preparation have subsided. When using a saturated solution of picric acid or mercuric chloride the structure of the muscle fibres becomes quite turbid, whitish and granular, on account of precipitation of protein. In case of formaline fixing the cross striation will again become visible after the initial "contraction". The ratio A:I will now be almost like the ratio A:I of contraction (table 1) but in places the structure of the fibres will appear more or less destroyed. A distinct

Preparation	Photo No.	Fixed by means of:	Mean Value			Coefficient of Variation		
			λ (in μ)	I (in μ)	$A + I$ (in μ)	V	$I + V$	$100 \times \frac{(I + V)}{V}$
136 IV	2221 γ 2225 γ	Formaline dissolved in distilled water (10 per cent)	1.47 1.10	0.90 1.01	2.37 2.11	3.0 8.3	2.2 3.3	2.2 7.1
136 XII	2304 α 2306 α	Formaline dissolved in Ringer solution (5 per cent)	1.43 1.48	0.85 1.45	2.28 2.93	3.3 4.0	2.6 3.5	2.6 1.5
136 VIII	2258 β 2261 β	Formaline dissolved in Ringer solution (5 per cent)	1.24 1.18	0.75 1.03	1.99 2.21	4.0 4.3	3.2 3.4	2.6 5.7
135 III	2163 α 2169 α	Chloral hydrate-glycerine (acc. to Studnitz)	1.42 1.93	0.84 1.58	2.26 3.51	2.2 7.2	2.0 5.7	2.1 3.1
131 III	1897 β 1903 β	Chloral hydrate dissolved in Ringer solution (10 per cent)	1.49 1.25	0.93 0.65	2.42 1.90	3.0 4.6	2.2 3.1	2.0 4.9
134 IX	2119 α 2123 α	Chloral hydrate-glycerine (acc. to Studnitz)	1.36 1.34	0.76 0.80	2.12 2.14	4.0 7.4	4.5 5.7	2.3 5.3
134 VI	2105 δ 2106 δ	Chloral hydrate dissolved in Ringer solution (10 per cent)	1.80 1.20	1.03 0.67	2.83 1.87	3.3 6.6	3.1 5.7	2.5 4.4

Table 1.

Fixing experiments with muscle fibres from m. glutens max. The fibres have been photographed and measured with the same optical system before and after fixing. (Magnification of photo 121 \times).

Preparations 134 VI and IX had been left for 24 hours in Ringer solution so that the fibres did not possess any contractility when the fixing was performed. The other preparations were contractile at the time of fixing.

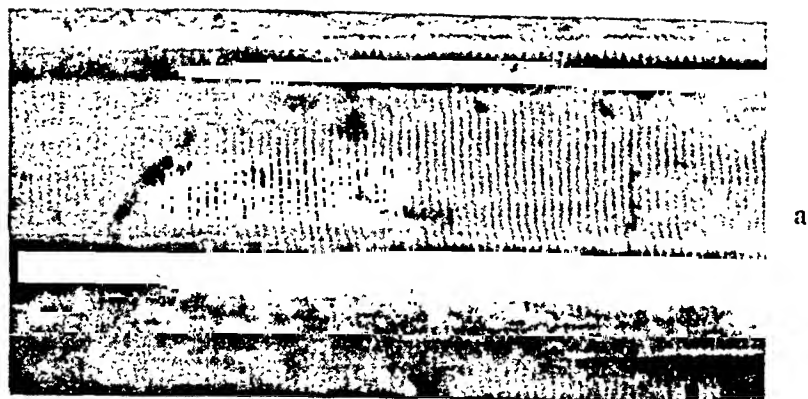
Fixatives used:

1 part formaline (abt. 36 per cent formaldehyde solution) + 9 parts distilled water.

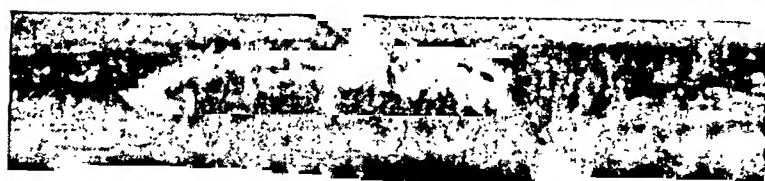
1 part formaline + 19 parts Ringer solution.

6 parts 10 per cent aqueous chloral hydrate solution + 1 part glycerine (acc. to Studnitz, 1935 a).

Ringer solution with 10 per cent chloral hydrate.



a



b



c

Fig. 5.

Muscle fibres from m. gluteus max. of the guinea pig. Magnification 400 \times .

a) Contractile muscle fibres.

b) Same fibres immediately after application of chloral hydrate-glycerine (6 parts 10 per cent aqueous chloral hydrate solution + 1 part glycerine). Muscle fibres intensely shortened with wrinkled sarcolemma.

c) Same fibres 2 minutes later. Irregular cross striation.

longitudinal striation with fibril bundles of various dimensions are often observed. *Stübel* (1920) has asserted that the best results as to the conservation of cross striation and uniformity of appearance is obtained by means of rapid fixing. Mammalian muscle fibres fixed by means of 5 per cent formaline-Ringer solution on the other hand exhibit greater uniformity than preparations fixed by means of 10 per cent aqueous formaline solution. By fixing with chloral hydrate-glycerine (composition as stated by *Studnitz*, 1935, a) or with chloral hydrate-Ringer solution (10 per cent chloral hydrate dissolved in Ringer solution) an initial "contraction" followed by a relaxation will likewise be produced, and in these preparations the cross striation also becomes altered and more varied than in vivo (table 1). Experiments in which mammalian muscle fibres were fixed by freezing with liquefied air and exsiccated in vacuum did not give better results, but likewise irregular cross striation and localized destruction of the structure.

Consequently no method exists according to which living, contractile muscle fibres may be fixed in their momentary functional state. The structural changes in the contractile substance of the muscle fibres produced by fixing is not the same — or at any rate not constantly the same — as the structural changes during contraction.

When fixing is postponed until the muscle fibres have lost their irritability the above mentioned changes in the contractile substance will be less pronounced (conf. table 1) and the preparations will exhibit a more uniform histological appearance. But of course the physico-chemical effects of the fixatives will, also in these preparations, cause changes. The tissue as a whole will shrink, the myofibrils particularly as to their cross dimension, so that the differences between sarcoplasm and myofibrils become more evident, granules appear, the cross membranes of the muscle fibres become more visible and the nuclei may become discernible. It is a question whether the detailed structural elements which are seen clearly in fixed and stained preparations are present in vivo, masked or latent, or whether they are merely artifacts produced by the fixing. A close comparison between living and fixed muscles is not sufficient for the solution of these problems. A detailed analysis of the structural changes caused in the muscles by the different physical and chemical agents and a more complete knowledge of the influence of the various methods of fixing

and staining would further the research work dealing with the details of the structure of muscles.

As mentioned above a number of investigations on the alterations produced in the muscles by chemicals is already available. Abnormal changes in the contractile substance may also be produced — and graduated — by electrical stimulation of muscle fibres (*Stübel*, 1920; *Ernst & Kellner*, 1936, b; *Speidel*, 1939; *Meneely*, 1939). By performing experiments on muscles fibres in vivo (*Speidel*, 1937—1939; *Meneely*, 1939) it has been possible to decide whether the changes produced are reversible or repairable. In the tail muscles of the tadpole in vivo *Speidel* could thus produce "contraction nodes" in which 5—20 compartments exhibited an intense contraction (shortening of 40 per cent or more). These contraction nodes were able to travel along the muscle fibres as peristaltic waves at a speed of 70 μ per sec. The changes were thus reversible. In case of more violent "contraction" of the fibres a "retraction cap" was formed in which the cross striations became concentrated and, in pronounced cases, disappeared completely, resulting in a homogeneous birefringent section in which the longitudinal striation had also vanished. The consistency of the tissue becomes harder, and the diameter of the fibre is often increased by these "retraction caps" or "clots". Thus we obtain a picture of a hyaline degeneration. Such structural changes have been described under many different names: waxy degeneration (*Zencker*, 1864), "maximal kontraktierte Wülste" (*Thoma*, 1906—1910), "simple, terminal, traumatic, waxy alteration" (*Jamin*, 1925, pp. 542—550) — to mention a few of them. What happens here is a contracture of the muscle fibres (conf. *Gasser*, 1930) which also corresponds to *Ramsey's & Street's* "δ state" (1940). As already mentioned it is impossible to distinguish between contraction nodes and retraction nodes. The latter are, however, irreversible, but repairable. In muscles in vivo they may be replaced by normal cross striation in the course of a few hours (*Speidel*, 1939). *Meneely* (1939) found that these changes are reversible as long as the cross striation in the retracted parts can still be distinguished.

Thus fixing of the muscle fibres may also give rise to misinterpretations within pathologico-anatomical research. When performing muscle biopsies it is far too frequently forgotten that an artificial hyaline degeneration may be caused

if the muscle fibres are fixed while still contractile. Fixing of living muscle fibres may also produce "granular disintegrations", "vacuolar degeneration" or "fibrillar splitting". Even if the fixing has been performed *lege artis* this kind of artifacts may occur at muscle biopsies, as it is impossible completely to avoid mechanical lesions of the tissue during the excision. There is no doubt that many cases of degeneration of the muscles described have actually been artifacts.

The muscle fibres are very vulnerable to mechanical influences; this is an experience gained in abundance when isolating living muscle fibres. At high degrees of stretch a displacement of the myofibril bundles parallel to each other may take place, at further extension a granular disintegration of the contents of the fibres will occur. The fibres can stand being stretched 100 per cent before the structure is destroyed. The displacement of the fibrils has, however, the effect that it becomes more difficult to measure the ratio A:I at higher degrees of extension.

If the sarcolemma is damaged a local contracture or "retraction node" is at once formed, see fig. 6. When the muscle fibre is cut through a "retraction cap" will at first be formed at the place of the lesion and the contents of the fibre will retract fairly rapidly and become wholly destroyed (*Thoma*, 1906—1910; *Fishback & Fishback*, 1932, and *Speidel*, 1939).

The stainability of muscle tissue.

When muscle fibres are fixed in a state of rest the anisotropic segments will always be stained more intensely than the isotropic segments and this is the case whether acid or basic stains are used. With regard to several stains, e. g. *Heidenhain's* iron alum hematoxylin, the difference between the stainability of A and I is not qualitative, but quantitative, as the boundary between cross striae of darker and lighter colours may vary according to the duration of the differentiating period during the staining — as emphasized i. a. by *Meigs* (1908) and *Hürthle* (1931, c). These conditions have been verified on mammalian muscles.

When dealing with muscle fibres fixed and stained while in the so-called "contracted" condition there is no agreement among investigators with regard to the stainability of the different cross striae. *Studnitz* (1934, 1935, a) has sum-

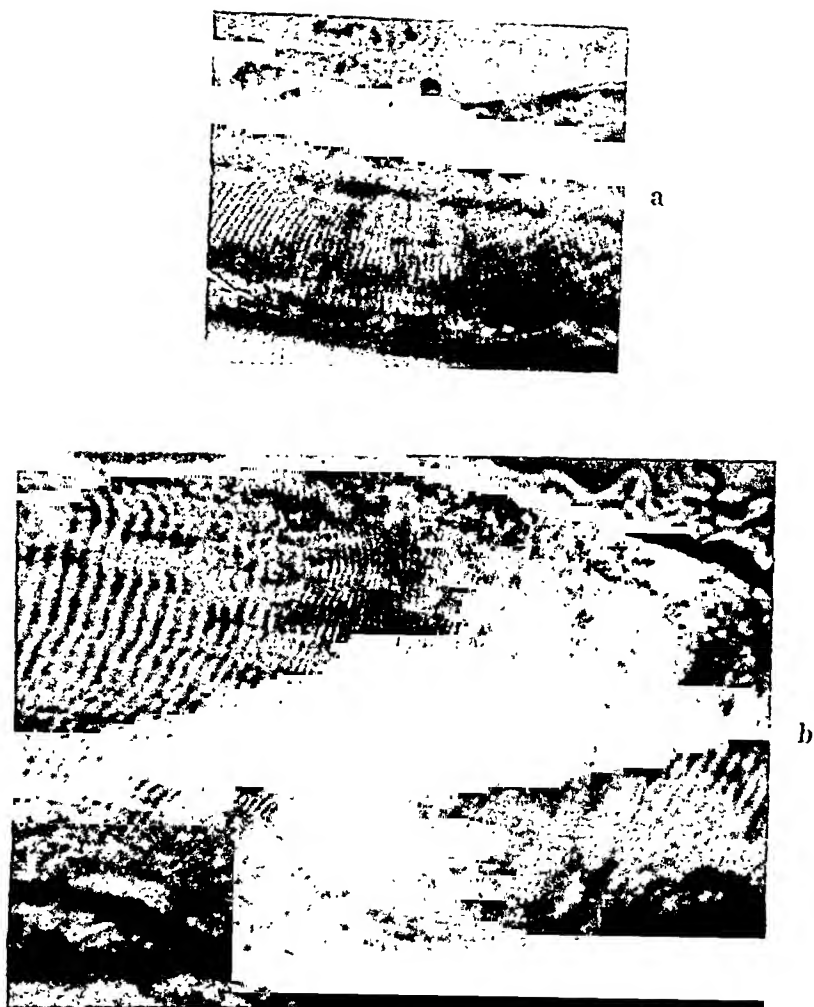


Fig. 6.

Injured muscle fibres from m. gluteus max. of the guinea pig.

a) Lesion of sarcolemma has produced localized "contraction" of the fibre contents. Magnification 400 \times .

b) Localized lesion with typical "retraction node". Magnification 750 \times .

marized the results of previous investigations, the usual conception having been that the stainability of A decreases while that of I increases during contraction, so that in contracted fibres the colour of I and C respectively is the most intense. This should apply to acid as well as to basic stains. *Studnitz*, (1935, a) himself, however, found that the stainability of A as well as that of I increases during contraction in such a way that the colour of A is always darker than that of I, only

in case of maximum contraction, when contraction bands (= C) are formed, may the stainability be reversed.

The interpretation of these alterations in the stainability is very difficult as there are so many factors which have a bearing upon the staining of muscles. Not only the myofibrillar structure, but also sarcoplasm and granules may be stained. The stainability further depends on the density of the tissue as well as on the electrostatic potential of the tissue (*Pischinger*, 1926, 1927; *Zeiger*, 1938, pp. 39—44), and the electrostatic potential may in turn vary according to the fixative used (*Zeiger*, 1930). Consequently parallelism between stainability and birefringence of the tissue is not always found. Staining of muscle preparations may thus give rise to misinterpretations. On the other hand there are many real structural elements which only become visible in stained preparations. It applies to staining as well as to fixing that only with a very thorough knowledge of the action of the various stains and fixatives is it possible to realise the actual character of the structure.

CHAPTER II.

METHOD AND MATERIAL.

A. Method.

The technique used for the isolation of living muscle fibres is worked out on the basis of the method used by *Brown & Sichel* (1930), *Asmussen* (1932—1936) and *Buchthal et al.* (1934—1946) in their experiments on muscle fibres of the frog.

Narcotization.

The animals used are narcotized with a 20 per cent aqueous solution of urethane, 1 cc. subcutaneously per 100 g. of body weight. By this method of application, narcosis, which occurs in the course of 1 hour, will generally last several hours and may be prolonged for more than 8—10 hours by a subsequent, smaller injection.

The direct or indirect irritability of the muscles is not influenced essentially by urethane narcosis, but urethane cannot be used in experiments in which an absolutely intact irritability of the motor end plates is required, e. g. in acetylcholine experiments. In that case chloralose narcosis is more convenient, the spinal reflexes being then preserved during the narcosis (*Dybing*, 1941, p. 59). In my experiments, in which exclusively electrical stimulation was used, chloralose narcosis held no special advantages. The survival of the excised muscle preparations proved to be no longer in case of chloralose narcosis than in urethane narcosis.

Gum arabic-Ringer solution.

Immediately after excision the muscle is placed in Locke's solution modified as follows:

NaCl -	8.5 g. per l.
KCl	0.42 g. per l.
CaCl ₂ (anhydrous)	0.24 g. per l.
Glucose	0.40 g. per l.
and gum arabic	60 g. per l.
+ NaHCO ₃	0.56 g. per l.
or polyviol Am.	20 g. per l.
+ NaHCO ₃	0.38 g. per l.

Gum arabic or polyviol Am. has been added to the solution to maintain the correct physiological colloido-osmotic pressure — about 350 mm. water column — and NaHCO₃ is added in a quantity which secures correct pH value (7.3—7.4) at 37—38°C. when a flow consisting of 99 per cent by volume of oxygen and 1 per cent by volume of carbon dioxide is constantly bubbling through the solution. The purpose of this constant addition of oxygen-carbon dioxide is in the first place to prolong the survival of the muscle preparations — by the admission of oxygen — and secondly to maintain the correct acidity of the solution — by means of the constant CO₂-tension.

Dialysis of the gum arabic solution.

The commercial gum arabic has an unknown content of salts — e. g. of potassium and calcium ions — it is therefore necessary to dialyze it.

150 g. gum arabic is dissolved by heating in a water bath in 1 litre Ringer solution of the following composition: NaCl 8.5 g. per l., CaCl₂ (anhydrous) 0.24 g. per l. and KCl 0.42 g. per l. By centrifuging particles not dissolved are sedimented and after decanting the solution is dialyzed in "Dupont de Nemon" cellophane bags for 30 hours against a Ringer solution with the same content of salts. During the dialyzing the external liquid is kept constantly agitated by allowing air to bubble through it, and it is repeatedly exchanged, an aggregate

amount of 30—40 litres of Ringer solution being used for dialyzing 150 g. gum arabic solution. The concentration of the gum arabic solution usually decreases from 15 per cent to 11—12 per cent during the dialysis on account of diffusion of water. This alteration of the concentration is determined by a Pulfrich refractometer and the solution of gum arabic is diluted with Ringer solution to such an extent that the final concentration of gum arabic is 60 g. per l.

Adjustment of the pH value of the gum arabic-Ringer solution.

pH-determinations are carried out by means of a glass electrode in connection with a "Radiometer" valve potentiometer, type P.H.M.3.

The gum arabic-Ringer solution has a fairly high acidity — $\text{pH} = 4.2 - 4.6$ — varying according to the stock solution of gum arabic used, for which reason it is necessary for every new stock to determine the amount of NaHCO_3 which must be added to the solution in order to obtain a permanent pH value of 7.3—7.4 at 37—38°C. during constant admission of oxygen-carbon dioxide; usually a concentration of 0.6 g. NaHCO_3 pr. l. is required. After addition of NaHCO_3 a flow of oxygen and carbon dioxide should be bubbled through the liquid for at least 5 minutes for the pH value to become constant. If the solution is left without addition of oxygen-carbon dioxide it becomes more alkaline; when the solution has been left at room temperature for 24 hours the pH value has been measured to be 8.0. The pH value changes slightly with changing temperature, a solution which has a pH of 7.20 at 20°C. has at 38°C. a pH of 7.35.

The required quantity of NaHCO_3 is not added to the solution until immediately before the latter is to be used, and the glucose, which serves as nutriment, is likewise added at this time, the quantity being only 0.40 g. per l.

Polyviol-Ringer solution.

Another substance of very high molecular weight, polyviol Am. (a chemically well-defined polyvinyl alcohol) is more convenient for these experiments than gum arabic. Polyviol Am. has a colloido-osmotic pressure of abt. 350 mm. water column in a 2 per cent solution, it does not require dialyzing.

but may be dissolved directly in the Ringer solution. Before NaHCO_3 is added the polyviol-Ringer solution usually has a pH value of 6.4—6.6. The NaHCO_3 concentration generally used is 38 mg. per 100 g.

Temperature control.

The temperature in the muscle chamber is measured thermo-electrically by means of a thermo-couple of special design and a sensitive galvanometer (*Buchthal, Høpncke & Lindhard*, 1944). The thermo-couple consists of a 0.5 mm. diam. cannula of stainless steel (V2a) to the point of which a 0.1 mm. diam. constantan filament is soldered — the constantan filament being insulated and passing through the hollow of the cannula. This needle thermo-couple is placed in the muscle chamber while the so-called "cold" junction is placed in a Dewar flask at a constant temperature, which is checked by means of a sensitive thermometer. The thermo-electric current is measured by a *Hartmann & Braun* moving coil light spot galvanometer: 5.7 of the scale units correspond to a temperature difference of 1°C . between the two junctions.

As far as possible the muscle preparations are kept at a temperature of $37\text{--}38^\circ\text{C}$. The rectal temperature of the guinea pig is about 38°C . The preparation dish with the excised muscle is placed in a electric thermostat (Heraeus type R. T. 360) and while the muscle fibres are prepared they are heated by radiant heat from two microscope lamps. The usual mechanical stage of the microscope has been replaced by a self-adjusting, heated stage (E. Leitz. 362).

Preparation technique.

After excision the muscle is placed in a shallow Petri dish with gum arabic-Ringer solution through which a constant flow of oxygen-carbon dioxide is passed. Two strips of cork are attached to the bottom of the Petri dish and the muscle is fixed by stainless insect needles to these strips. Under a binocular microscope, magnification $28\text{--}70\times$, a small bundle of muscle fibres is first isolated by means of a pair of modified iridocyclitis scissors (designed for this purpose by *F. Buchthal*, see *Lundin*, 1944, p. 11), a pair of delicately pointed tweezers and various fine preparation needles, i. a. a metal wire, dia-

meter $100\ \mu$, with a sharply ground point and attached to a glass tube. While performing the preparation it is convenient to support the hands on a couple of sand bags. The muscle fibres are sensitive to even light pressures — a slight touch with a needle may be sufficient to destroy the structure. Only at the tendons it is possible to catch hold of the muscle fibres without causing any damage. Care must likewise be taken never to expose the fibres to excessive tension. Consequently the preparation cannot be done merely by carefully removing strands and fibrils of connective tissue by needles, it is also necessary to cut them by scissors. When removing the muscle fascia and coarser strands of connective tissue it is most convenient to use direct illumination; for the subsequent isolation of the smaller fibre bundles transmitted illumination is, however, required. When isolated, the fibre bundle is transferred to the muscle chamber (fig. 7) where two adjustable metal

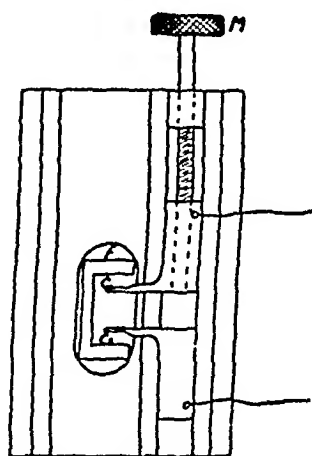


Fig. 7.

Muscle chamber. (Acc. to Buchthal & Knappeis, 1940).

C_1 and C_2 : adjustable metal clamps for attaching the tendon ends of the preparation.

M : micrometer screw for adjusting one metal clamp.

F : celluloid frame secured to the slide and constituting the three walls of the muscle chamber.

clamps fix the tendon ends to the slide; the clamps are insulated and by means of sockets they may be connected to wires from an electric stimulator. By means of a micrometer screw one of the clamps may be adjusted very accurately so as to stretch and relax the fibres. The final and most delicate

preparation procedure is performed after the preparation has been placed in this chamber — also under a binocular microscope and with transmitted illumination. Then the preparation is covered with a cover glass and as the slide is fitted with a 0.45 mm. high celluloid frame the preparation is thus enclosed in a chamber filled with gum arabic-Ringer solution. A little lanolin or vaseline on the celluloid frame will prevent the Ringer solution from escaping. During the actual experiment the chamber is secured to the heated stage of the microscope and consequently kept heated as described above.

Optical system and photographic technique.

For the photographic recording the following objectives have been used:

Objective	Zeiss B	16 ×	(NA: 0.40).
—	-	Epi 40 ×	(NA: 0.65).
—	-	D 40 ×	(NA: 0.65).
—	-	70 ×	apochromatic, water immersion (NA: 1.25).

The Zeiss objective Epi 40 × is particularly suitable for the investigations described, i. a. on account of its large free working distance.

Microphotography has been performed either by means of a Zeiss "Kolibri Phoku" camera (eyepiece 10 ×) or by a Leica camera with Leitz "Micro-attachment" with compensating eyepiece 10 ×. The negative films used are Agfa tone negative film "Special", and Ilford "Selo Hypersensitive" which are both of them fine grain films giving relatively sharp boundaries (conf. *Buchthal, Knappeis & Lindhard*, 1936). The magnification of the negative films will appear from table 2.

Objective		Magnification of Photo		
		16 ×	40 ×	70 ×
Leica	(Eyepiece 10 ×)	46 ×	121 ×	236 ×
Phoku	(Eyepiece 10 ×)	77 ×	202 ×	395 ×
Fall camera	(Eyepiece 9 ×)		72 ×	

Table 2.

Magnification of object on negative films with the various optical systems used.

The light source has been a "Universal Microscope Lamp Lux S" according to Romeis with an Osram focus lamp, type 2G, and an Osram high pressure mercury lamp, type HBO, with a horizontal candle power of 3000 Hefner candles and a luminous flux of abt. 25,000 lumen. The latter had to be placed in a box behind a thick plate of safety glass as a protection against explosions and was cooled by a continuous flow of air from a vacuum cleaner. After having passed a container with cooling water the light is concentrated on the plane mirror of the microscope by means of two converging lenses of 5 dioptries and a diameter of 14 cm. The microscope was fitted with a centred Zeiss condenser (NA: 1.4). It was frequently more convenient to use the condenser without front lens. A light source of high intensity was required as exposures longer than 1/25—1/10 sec. resulted in blurred pictures in the contraction experiments.

Measurement.

The microphotographs are measured on the negative films by means of an eyepiece screw micrometer (C. Zeiss). The cobwebs of the eyepiece can be adjusted by a micrometer screw and the movements can be read on the graduated barrel. The eyepiece micrometer is fitted with an adjustable front lens to be able to focus the cobwebs and the image of the object without parallax. The magnification of the micrometer eyepiece is $7\times$. It is used in connection with a Zeiss objective with a magnification of $2\times$, the total magnification used thus being $14\times$. The total magnification used for measurements and photographs appears from table 3.

Objective	1 μ ∞ divisions		
	16 \times	40 \times	70 \times
Measured directly	2.47	6.47	12.41
Measured on Leica photo (Eyepiece 10 \times)	15.7	41.0	80.2
Measured on Phoku photo (Eyepiece 10 \times)	26.3	68.7	134.5
Measured on fall camera photo (Eyepiece 9 \times)		24.6	

Table 3.

Total magnification of the object when measuring directly or on the various negative films.

For each muscle fibre ten A- and ten I-layers are measured, that is altogether ten compartments. The layers measured are, as far as possible, adjacent layers whereby more accurate results are obtained and the work is facilitated because the number of readings is reduced. Accuracy of measurements and control experiments are dealt with in chapter III.

Electric stimulation.

The electric stimulation is performed either by faradic current from a Dubois-Reymonds inductor with a primary voltage of 4—6 V. or by rhythmic discharges from a condenser in an apparatus designed by *Lange* (1931); rectangular current impulses of a duration of 1 msec. from a thyatron generator have also been used — especially for experiments on single contractions. To obtain tetanic contraction of mammalian muscles frequencies of more than 80 cycles per sec. are required (conf. chapter V). The strength of the stimuli is chosen so as to obtain maximum single contraction, but as near as possible to the threshold value in order to avoid unnecessary lesion of the muscle fibres.

The stimulation is transmitted to the two tendon ends of the preparation through the adjustable metal clamps of the muscle chamber; local stimulation is also applied by specially designed micro-electrodes consisting of a 40 μ diam. nickel-chromium wire fitted in a pointed capillary tube, the wire being slightly retracted within the opening of the capillary. A small amount of Ringer solution will penetrate into the capillary producing the required contact. By means of a micro-manipulator the electrodes are placed on the isolated fibre bundle or individual fibre. When such local stimulation is employed the stimulation of the muscle fibre is direct, otherwise it has always been indirect — i. e. via motor nerve fibres or motor end plates.

Investigation on muscle fibres in situ.

For the investigations on muscle fibres in situ m. obliquus abd. intern. has been used. During these experiments the narcotized guinea pig is placed on its back on a small operating table in front of the microscope so as not to interfere with the illumination of the microscope. The stage of the microscope

is replaced by a 4 by 4 cm. object support with rounded edges and fitted with a large illumination diaphragm. It is covered with a glass plate with a central, cylindrical glass chamber 5 mm. high and 12 mm. in diameter, serving as a support for the isolated muscle bundle. The abdominal wall of the guinea pig is opened by a U-shaped cut following the linea alba, proceeding from there laterally, partly below the curvature and partly proximal to the symphysis. In beforehand the skin, *m. obliquus abd. extern.* and the coarser strands of the superficial fascia of *m. obliquus abd. intern.* have been removed from a small circular area of the lateral part of the abdomen. The abdominal wall is folded back, the exterior surface of the abdominal wall resting on the stage; in this way the exposed part of *m. obliquus abd. intern.* is made to rest across the cylindrical glass chamber. The flap of the abdominal wall is secured by means of a pair of clamps placed in the two holders of the micro-manipulator. By these holders it is moreover possible to adjust the preparation as required, stretch and relax the muscles etc. Except at the circular area, the abdomen and the internal surface of the opened abdominal wall are covered by paper lint moistened with gum arabic-Ringer solution. *M. transversus abd.* is removed from this area and the fibres of *m. obliquus abd. intern.* are excised till only one layer remains, often a small fibre bundle is completely isolated. The preparation is kept moist by continuous instillation of gum arabic-Ringer solution of body temperature. The muscle bundle is kept warm by a carbon filament lamp. Electric stimulation is applied by micro-electrodes fitted in the two other holders of the micro-manipulator.

In such preparations the abundant vascularisation of the muscle with capillary loops along the individual muscle fibre appears very clearly. By working very carefully and when not attempting to isolate very small fibre bundles it is possible to keep the circulation completely intact, but this can only be done at the cost of the distinctness with which the cross striation is seen. Hemorrhage causes a good deal of difficulties in these experiments, but by avoiding larger vessels and by frequently rinsing with gum arabic-Ringer solution it is possible to keep the field fairly free from blood cells.

This method is more difficult than isolation of muscle fibres from excised muscles. It may f. inst. prove rather difficult to keep the preparation in a sufficiently fixed position

as respiratory movements are often transferred to the preparation in a purely mechanical manner and perhaps also on account of co-innervation. Further, it is more difficult to obtain completely reproducible and well-defined experimental conditions, f. inst. for the determination of the equilibrium length of the individual muscle fibre. For this reason the method is not used as standard method, although the muscle fibres must be said to function under more "natural" conditions than when excised.

Measurement of the birefringence of muscle fibres.

For the quantitative determination of the birefringence of muscle fibres a Babinet's quartz wedge compensator is used. By means of this apparatus it is possible to measure the phase difference which will occur when the light passes the muscle fibre which is placed between two nicols (*Ambrohn & Frey*, 1926, p. 60). When the diameter of the muscle fibre is also measured the birefringence may be calculated from the formula: $(n_a - n_o) = \frac{\gamma\lambda}{d}$. The birefringence $(n_a - n_o)$ is expressed by the difference between the refractive indices of the anisotropic substance for the extraordinary ray (n_a) and the ordinary ray (n_o). d denotes the thickness of the object, λ the wave length of the light employed and γ the phase difference between the two rays. The phase difference measured ($\Gamma = \gamma\lambda$) is expressed in $m\mu$, the value of the wave length employed in $m\mu$ (for green light $\lambda = 546 m\mu$) being substituted in the formula. The phase difference is thus proportionate to the birefringence and the thickness of the object. In the Babinet's compensator eyepiece two equal-angled quartz wedges may be moved in relation to each other so that they form a disc with plane-parallel sides of variable thickness. The axis of one wedge is at right angles to the edge, the axis of the other is parallel to the edge. If the Babinet's compensator is placed in a diagonal position between the crossed nicols a dark interference line is seen at the place where the two quartz wedges are of the same thickness, thereby compensating the phase differences of each other. If a birefringent substance, e. g. a muscle fibre, is placed on the stage in a diagonal position to the two nicols and parallel to the Babinet's compensator the interference line will be displaced proportionate to the

phase difference of the object, to the right or to the left dependant on whether the anisotropy of the object is positive or negative. The deviation of the interference line is compensated for by altering the adjustment of the compensator eyepiece, i. e. altering the thickness of the plane-parallel quartz disc; in this way a measurement of the phase difference is obtained. To obtain absolute figures — in $m\mu$ — for this value the apparatus is calibrated by means of objects of known phase difference — i. e. selenite plates the thicknesses of which correspond to $1/4$ — $1/2$ —1 and 2λ . The fact that the muscle fibre consists of alternate layers of isotropic and anisotropic substances does not influence the measurements as the Babinet's compensator gives a constant phase difference for an infinitely thin segment of the object.

Fig. 8 shows the optical arrangement (conf. *Buchthal & Knappeis*, 1938). The light source is the previously mentioned high-pressure mercury lamp. The monochromatic light chosen

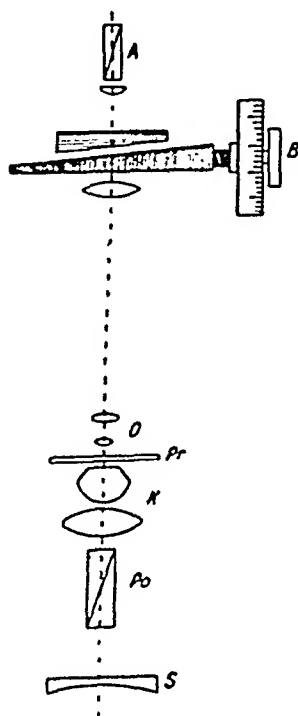


Fig. 8.

Optical arrangement for birefringence experiments (acc. to *Buchthal & Knappeis*, 1938).

A and Po: crossed nicols, analyser and polarizer respectively.

B: Babinet's compensator eyepiece. O: objective.

K: condenser. S: microscope mirror. Pr: preparation.

is that of the green mercury line corresponding to $\lambda = 546$, a green glass filter (Schott u. Gen., Jena) and two filters, one with a solution of didymium nitrate the other with a solution of tartrazine, being inserted between the light source and the mirror of the microscope. The wave length is checked by spectroscopy. On the microscope the polarizer (Zeiss Polariser I) is placed in the aperture of the diaphragm of the substage condenser, the analyser (Zeiss Analysator A) is secured on top of the eyepiece lens of the Babinet's compensator, the two nicols being at right angles to each other. The Babinet's compensator (Winkel-Zeiss) is in a diagonal position to them, and the muscle preparation is placed in the previously described chamber on the adjustable mechanical stage. As the optical axis of the muscle fibre is parallel to the fibre axis the preparation should be placed in a diagonal position to the nicols, but parallel to the axis of the Babinet's compensator. During these experiments it is necessary to work with an accurately centred optical system and it has therefore been an advantage that the condenser was fitted with a centring adjustment. The objectives used were the Zeiss B 16 \times and the Zeiss Epi 40 \times . Neither the objectives nor the gum arabic-Ringer solution in the chamber exhibited any depolarization phenomena.

For these experiments isolated, single muscle fibres and marginal fibres from isolated muscle bundles have been used and, as mentioned before, always intact, contractile fibres. A single muscle fibre produces a curved deviation of the interference line in the Babinet's compensator corresponding to the cross section of the fibre, the greatest deviation occurring at the point where the fibre is thickest. When examining fibre bundles only marginal fibres from which superimposed fibres have been removed for at least two thirds, have been used, so that it has been possible clearly to recognize the maximum deviation of the interference line. The phase difference has always been determined three times for each single fibre, each result is thus the mean figure of three measurements. With the experimental arrangement employed one division of the scale of the Babinet's compensator corresponds to a phase difference of $0.924 \text{ m}\mu$.

The thickness of the fibre and the height of the compartment are measured by means of the eyepiece micrometer (Zeiss) with such a magnification that one division on the

micrometer barrel corresponds to 0.405 and 0.155 μ respectively. When measuring fibre thickness the plane in which the fibre appears widest and with absolutely distinct edges is brought into focus. As an expression of the degree of stretch of the fibre the length of ten compartments has been measured. When determining fibre thickness and height of compartment three different readings have likewise been taken for each experiment.

Determination of the birefringence of the individual structural elements of muscle fibres.

For qualitative determinations of the birefringence of the individual structural elements almost the same apparatus is used as for the quantitative determinations. During these experiments the analyser is placed in the tube of the microscope and the two nicols are secured at right angles to each other while the preparation, accommodated in the chamber on the rotating, mechanical stage, is adjusted in diagonal position, the position in which the muscle fibres exhibit the highest light intensity. For these investigations monochromatic light (green, $\lambda = 546$) is also used. The preparation is photographed in the usual manner, but as the light intensity is here necessarily lower, the time of exposure cannot be reduced to such an extent that the fibre may be photographed during contraction in polarized light.

Recording of the changes in the ratio A : I during single contraction as a function of time.

For measuring the changes in the ratio A : I of the muscle fibre as a function of time during single contraction *Buchthal's & Knappeis'* experimental apparatus (*Buchthal & Knappeis*, 1943, a) is used. A photographic recording of 100 pictures per sec. with an exposure of less than 0.4 msec. can be obtained by this arrangement.

The light source employed is a water cooled high-pressure mercury lamp (Philips "Philora", 500 V.) with a brightness of 33,000 stilb. and a luminous flux of 15,000 lumen. The light passes a water cooled collector with two lenses of 20 dioptries and after further cooling it is focused on a rotating disc by a converging lens. The disc, which is placed in front

of the condenser of the horizontally arranged microscope, has a diameter of 22 cm., it has two slits and is rotated by a synchronous motor at a speed of 3,000 r. p. m. hereby producing the above mentioned rate of pictures and time of exposure. A Zeiss Epi objective $40\times$ and a projection eyepiece $9\times$ is used, and by means of a fall camera placed at a distance of 5 cm. from the front lens of the eyepiece a 30 cm. long film strip (Agfa tone negative film, "Special") is moved past the front lens of the eyepiece at a speed of 1 m. per sec., so that 30 pictures may be recorded on each film. The magnification of the negative films is $72\times$. By means of a special contact mechanism the electrical stimulation is started immediately when the film begins to move. In these experiments the muscle fibres are stimulated with rectangular current impulses of a duration of 1—2 msec.

Investigation of the mechanical properties of the muscle fibres.

To investigate the mechanical properties of muscle fibres an experimental arrangement similar to that of *Buchthal* (1942, pp. 6—19) and *Buchthal & Kaiser* (1944) is used. By this arrangement it is possible simultaneously and continuously to record length, tension and stiffness of the fibres. The stiffness is examined by measuring the variations in tension caused by periodical variations of the length of the fibres ($\Delta\text{tension}/\Delta\text{length}$).

The experiments are performed on small bundles of muscle fibres, 0.1—1 mm. in diameter with an equilibrium length of 4—12 mm. and a weight varying from 5—12 mg. and containing from 20—200 muscle fibres. The preparations employed have always consisted of parallel fibres of uniform length with the fascia removed and without tendinous tissue embedded in the bundle.

During the experiments the preparation is placed in a chamber filled with polyviol or gum arabic-Ringer solution (fig. 9). The chamber is surrounded by a jacket through which water of a suitable temperature flows to keep the temperature of the chamber constant at $37\text{--}38^{\circ}\text{C.}$; the temperature is checked thermo-electrically.

The tendon ends of the fibre bundle are fixed by two pairs of stainless steel micro-tweezers of special design. One of these transmit tension by means of a condenser myograph

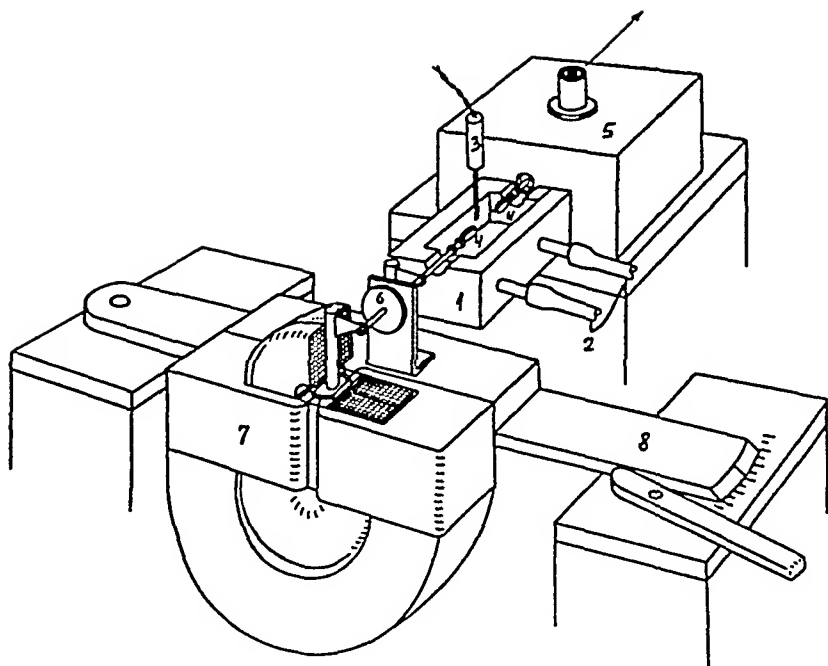


Fig. 9.

Experimental arrangement for recording the mechanical properties of muscle fibres (acc. to Buchthal, Kaiser & Knappeis, 1944).

- 1) muscle chamber. 2) pipes for circulating water of suitable temperature through the jacket of the muscle chamber. 3) thermo-needle for measuring temperature in muscle chamber. 4) micro-tweezers for attaching the preparation. 5) condenser myograph. 6) condenser for recording the amplitudes of the variations in length. 7) electro-magnetic system for producing periodic variations in length of the muscle preparation transmitted through one of the micro-tweezers. 8) adjustable slide for coarser adjustment of stretch and relaxation of muscle fibres.

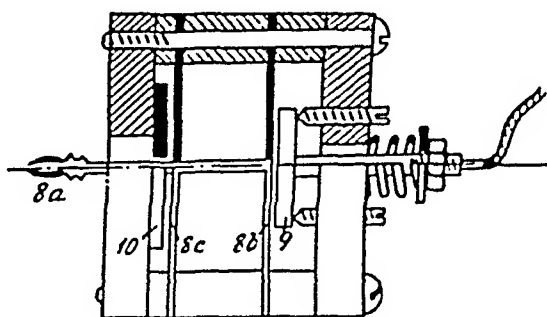


Fig. 10.

Condenser myograph (acc. to Buchthal, Kaiser & Knappeis, 1944).

- 8 a) micro-tweezers. 8 b and c) phosphor-bronze springs securing parallel movements of the micro-tweezers. 9) adjustable condenser electrode which together with 8 b constitutes a condenser. 10) condenser electrode with electric field for mechanical calibrating.

(fig. 10), the minute displacements of two condenser plates caused by the tension of the attached muscle fibres being converted into variations in capacity. Two spring plates of phosphor-bronze keep the micro-tweezers in position, allowing only slight movements in a longitudinal direction. One of the phosphor-bronze plates acts as an earthed condenser plate which is removed slightly from the other, fixed condenser plate during stretch or contraction of the fibre bundle. The variations in capacity of these two plates, which are screened against external disturbances of the capacity, are by means of a high-frequency circuit (acc. to Zacharias, 1938) converted into variations in potential and are amplified by a two-stage d. c. amplifier for the recording of static tension and by an a. c. amplifier with a higher amplification factor for recording the variations in tension (fig. 11). The variations in potential

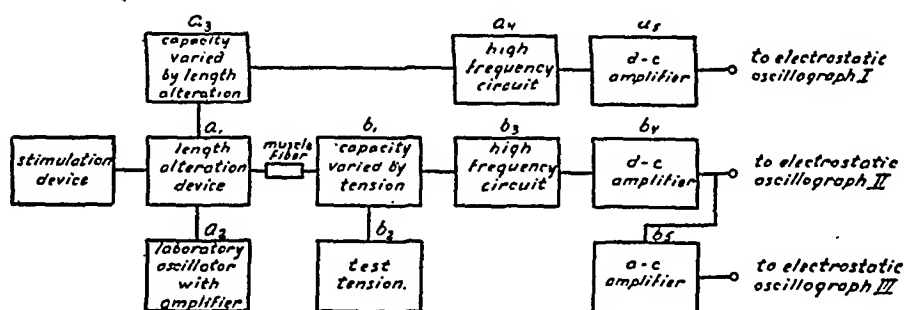


Fig. 11.

Diagrammatic reproduction of the experimental arrangement for investigating the mechanical properties of muscle fibres (acc. to Buchthal, Kaiser & Knappeis, 1944).

are recorded by electrostatic mirror oscillographs capable of reproducing frequencies up to 3000 cycles per sec. The oscillographs used are slight modifications of the type designed by Beer and employed in sound film technics. A thin reflecting band of light metal (0.0015 mm.) is stretched between two sets of solid electrodes a small distance from each other. The reflected ray falls upon photographic paper which is drawn through the camera by a synchronous motor.

The sensitivity of the condenser myograph is uniform for frequencies from 0 to 800 cycles per sec., and the deflection of the electrostatic oscillograph is proportionate to the variations in potential. By means of a calibrating device the sensitivity and linearity of the system may be checked. Dis-

turbances caused by mechanical vibrations have been eliminated by mounting the experimental apparatus on concrete blocks, and control experiments have secured that no errors are caused by acoustic oscillations.

Periodical longitudinal oscillations may be imposed upon the other pairs of micro-tweezers securing the fibre bundle, the tweezers being connected to the electromagnetic system of an ink-writer unit otherwise used for electro-encephalography (fig. 9). The oscillations of the electromagnetic system is produced by an oscillator and the frequencies used are 10 and 100 cycles per sec.

The equilibrium length of the fibre bundle is determined either by a measuring microscope or by a pair of compasses. Larger variations in the length of the fibre bundle is obtained by moving the micro-tweezers, the latter and the electromagnetic oscillating device being both attached to a movable slide. The movements may either be read directly from a graduated scale or may be recorded photographically by means of an electric amplifying system in connection with an electrostatic oscillograph. It is thus possible simultaneously to record tension, variation in tension and variation in length by means of three different oscillographs. The record is measured by a measuring microscope, the movable tube and stage of which make it possible to measure the curves in both planes with an accuracy of 0.01 mm.

For simultaneous recording of length, tension and dynamic stiffness the condenser myograph used by *Buchthal* for his first mechanographic experiments (1942, p. 9) is employed. The micro-tweezers transmitting tension are more mobile in this case as they are held by thin steel springs. To maintain constant sensitivity, i. e. to keep the distance between the two condenser plates constant, the movable condenser plate is provided with a coil placed in the air gap of a powerful permanent magnet, exactly as in an electrodynamic loudspeaker. The stronger the current which flows in the coil the higher the mechanical tension will be with which the coil is drawn into the magnetic field. The current in the coil which results in unchanged capacity in the condenser system, i. e. unchanged distance between the condenser plates, is a measure of the static tension of the muscle fibre bundle.

In a few experiments, e. g. for determination of the variation of the tension during single contraction, the condenser

myograph has been used in connection with a cathode ray oscillograph with a condenser-coupled amplifier (Philips type G M 3156) with »single stroke« time base. The oscillograms are photographed by means of a Contax camera, the lens of which was an "Astro Ardenne Special F. 1", and the negative film is measured by means of the measuring microscope.

The electric stimulation of the muscle fibres is transmitted through the two steel micro-tweezers and is, in these experiments, produced by a thyratron stimulator which allows for variations of the shape, voltage, duration and frequency of the current impulses. Rectangular current impulses of 1—2 msec. are used.

B. Material.

In order to lend itself to isolation of individual muscle fibres a muscle must fulfil the following requirements: a) tendinous origin and tendinous insertion, b) suitable length of fibres, c) uncomplicated structure, d) small amount of connective tissue, e) easy accessibility for excision.

a) While isolating the muscle fibres the sarcolemma should be kept intact in the whole length of the fibre. If the sarcolemma is cut through or otherwise injured local, irreversible changes in the contents of the fibre will immediately occur, and the changes will quickly spread throughout the fibre (conf. *Jamin*, 1925, pp. 542—550; *Speidel*, 1937—1938). The muscle fibre is very sensitive to pressure and to more pronounced stretch; it cannot sustain the grasp of a pair of tweezers. For this reason it is convenient to use muscles with tendinous origin and insertion. In that case it may be possible to avoid cutting through the fibres during the excision, and the small piece of tendon which may be preserved in connection with each isolated muscle fibre will be convenient for gripping the fibre.

b) It is technically difficult to isolate fibres the length of which exceeds 2—4 cm. It is of course possible to isolate only a part of their length, the remaining being protected by the other fibres of the bundle. But the method has several drawbacks in spite of the greater durability of the preparations obtained. The thick bundles at the ends of the preparation

will require a higher muscle chamber the result of which may be that the working distance of the objective is exceeded. The isolated part of the fibre may not be in the same plane throughout its length resulting in defective focussing. It will not be possible to adjust the tension or degree of stretch of such preparations within sufficiently narrow limits due to the greater tendency of the non-isolated parts to adhere to slides or cover glasses. Finally it is more difficult to obtain "pure" isometric contractions of such preparations as varying co-contractions and variable adhesion of the thick non-isolated parts of the fibre bundle will interfere. Nevertheless, it has often been infeasible to isolate more than a part of the length of the muscle fibres, the mammalian muscles being so difficult to work with that isolation of muscle fibres in toto approaches the technically impossible.

c) It is hardly necessary to explain that a complicated muscular structure impedes the preparation work. It is of course most easy to obtain good results if the fibres of the muscle run parallel from tendon to tendon. Flat muscles consisting of thin layers are likewise preferable to more solid and thick ones.

d) The most important consideration is to the connective tissue of the muscle. In cross striated mammalian muscles the amount of connective tissue is fairly ample — compared with conditions in frog muscles — and the strands of connective tissue seem to be much tougher and stronger in proportion to the muscle fibres than it is the case in muscles of the frog. It is a contributing cause that the muscle fibres, at any rate from the muscles of guinea pig, cat, rabbit and goat used, are on the whole thinner than those of the frog muscles. The consequence of these circumstances is that while it is quite feasible, when isolating frog muscle fibres, to pull the muscle fibres slightly in order to loosen small fibrils of connective tissue, such a method would in mammalian muscles result in overstraining and damaging of the muscle fibres, while the fibrils of connective tissue would remain unbroken. When isolating mammalian muscle fibres it is therefore necessary to use cutting instruments to a much greater extent.

In case of frog muscles it is often preferable to leave the isolated muscle for 24 hours in gum arabic-Ringer solution at a low temperature (1—4°C.); the electric irritability of the muscle is not altered essentially and the frequent spontaneous

contractions, often met with in freshly isolated frog muscle fibres which may be rather inconvenient, are avoided and — a fact which should be particularly emphasized in this connection — a slight autolysis sets in which facilitates the separation of the connective tissue from the actual parenchyma of the muscle. It is not possible to utilize a similar autolysis in the case of mammalian muscle fibres. If they are excised in toto they remain contractile for at most 2—4 hours, even when kept under optimal conditions, and in the course of 2—4 hours the autolysis will not proceed sufficiently to facilitate isolation. It is also impossible to trace such effects of autolysis when the muscle is allowed to remain in situ for some time after the death of the animal. In situ the survival of mammalian muscles is even shorter, $\frac{1}{2}$ —2 hours at most (conf. *Tryde*, 1861, pp. 6—14).

It is not only the amount of connective tissue, but also the innervation of the muscle which should be taken into consideration. The nerve branches with their sheaths are very tough and they are intimately attached to the muscle fibres at the motor end plates. This again means that muscles with highly coordinated movements which have relatively few muscle fibres per motor unit (*Clark*, 1931) and consequently abundant innervation are especially difficult to use when isolating muscle fibres, e. g. it proved impossible to isolate muscle fibres from the external eye muscles.

e) On account of the relatively short survival of the cross striated mammalian muscles it has proved convenient to keep the test animal permanently narcotized and excise suitable muscles in the order they are required. In this way “fresher” material is obtained and fewer test animals consumed. On the other hand the excision must not be too mutilating. The muscles to be excised must be easily accessible.

In order to find out which muscles satisfy all these requirements and which will consequently afford suitable experimental material various test animals: white mouse, rat, guinea pig and cat have been examined and dissected*). It soon appeared that muscles from white mice and rats were too small to be convenient. The muscles of the guinea pig were, on the other

*) The myology of the rodents has i. a. been described by *Parson* (1894—96). The anatomy of the rat by *Green* (1935), the anatomy of the rabbit by *Krause* (1884) and by *Gerhard* (1909) and the anatomy of the cat by *Reichard & Jennings* (1930).

hand, of a suitable magnitude, the guinea pig has consequently supplied the vast majority of the material used for the investigations described in the following. The muscles used were *m. gluteus max.*, *m. serratus post.* and *m. obliquus abd. intern.*

In the guinea pig *m. gluteus maximus* is not a particularly powerful muscle, it is only a few mm. thick, triangular, almost equilateral, its length is approximately $1\frac{1}{2}$ —3 cm. The arrangement of the fibres is fanshaped, the fibres converging from the extensive, flat tendon of origin from the iliac crest and the sacrum to the short, but strong tendon of insertion at trochanter tertius femoris. On the superficial surface the fascia is fairly strong, but on the deep surface it is only thin. Vessels and nerves enter the muscle from the deep surface, slightly proximal to the caudal edge of the muscle. The muscle may easily be excised without hemorrhage of any importance, as it is superficially situated. The caudal bundles have especially been used — the course of the fibres being parallel from tendon to tendon in this part of the muscle.

M. serratus posterior consists of very thin layers of muscle fibres of a length varying from $\frac{1}{2}$ —2 cm. In the individual slips the course of the fibres is practically parallel and the muscle as a whole only contains little connective tissue. When excising the muscle it is somewhat difficult to get enough tendinous tissue at the end of the fibres where the slips are attached to the ribs, but the muscle is fairly easily accessible; by cutting through the superficial flat dorsal muscles and pushing aside the scapula *m. serratus post.* will become uncovered.

In the guinea pig the length of *m. obliquus abd. intern.* is up to 4—10 cm. measured along the fibres — obliquely medially — ventrally — proximally. Actually this muscle is not particularly well suited for isolation of excised muscle fibres, but it is very convenient for examination of muscle fibres *in situ*. It has been chosen in preference to *m. obliquus abd. extern.* because the connective tissue of the latter is more abundant and the fascia more tightly attached in *m. obliquus abd. extern.* than in *m. obliquus abd. intern.*

Among the muscles of the rabbit *m. serratus post.* is the most convenient. Most of the muscles of the cat have the drawback that the muscle fibres are too long. The most suitable experimental material from the cat is *m. serratus post.* *M. transversus thoracis* (sive *triangularis sterni*) is also of a

suitable size, has parallel fibres and a small amount of connective tissue, but the position of the muscle on the inside of costae and sternum renders it rather inaccessible. In muscles of more complicated structure there may be fibres of convenient size and tendinous origin and insertion — among these may be mentioned: *m. flexor carpi radialis*, *m. flexor digitorum sublimis* and *caput laterale gastrocnemii*. In these cases, however, the isolation of the primary fibre bundles is more elaborate.

For the purpose of orientation an analysis of the shape and size of the muscle fibres of the above mentioned muscles has been performed. Numerous methods are described for maceration of the connective tissue for such investigations. The muscles may e. g. be treated with solutions of acetic acid, osmic acid, caustic potassium or the like. For the present investigations *F. C. C. Hansen's* method has been used, it is recommended by *Lindhard* (1926) as being convenient and suited for the purpose. The muscle is boiled for 2 hours in tap water and to avoid excessive shortening of the fibres the muscle is left in situ by simply boiling the whole guinea pig. After boiling the connective tissue is completely dissolved and the individual muscles may easily be withdrawn and, with some care, split into bundles and fibres.

Table 4 gives the result of a number of measurements of primary bundles and fibres from *m. gluteus max.* of an adult guinea pig, weight abt. 750 g.; the length of the muscle from origin to insertion at the proximal and distal edge was 25.0 and 18.5 mm. respectively. The aponeurosis at the origin was 19.0 mm. wide. The vast majority of the muscle fibres of *m. gluteus max.* pass from tendon to tendon, their ends being blunt and rounded. The few muscle fibres with free ends, which have been found, have been fusiform with tapering endings. Isolated living muscle fibres have shown no variation in thickness along the isolated length, but by the above mentioned method (boiling in water) it has been found that the thickness of the individual fibres varies throughout the length of the fibre, the observation also applying to fibres which pass from tendon to tendon. This finding is no doubt to a great extent caused by the method of preparation, as a corresponding variation in the height of the compartments has been measured, and in some parts of the fibres the cross striation is actually destroyed.

Length of Fascicles (in mm.)	Length of Fibres (in mm.)	Thickness of Fibres (in μ) Height of Compartment (in μ)	Shape of Fibre Endings
12.1	12.0	71 — 67 — 60 — 71	blunt
	8.0	24 — 55 — 66 — 44	tapering
	12.2	40 — 48 — 40 — 49 — 48 2,05 — 2,27 — 2,29 — 2,09 — 2,15	blunt
	12.2	48 — 39 — 45 — 47 2,29 — 2,06 — 2,15 — 2,15	blunt
	12.0	59 — 52 — 55 — 41 — 51 2,22 — — — 2,06 — — 2,10	blunt
16.4	15.1	60 — 45 — 48 — 46 2,06 — — — 1,98	blunt
11.9	11.0	46 — 47 — 48 — 43 — 43 1,98 — — — 2,02 — — 2,15	blunt
	11.5	62 — 64 — 61 — 61 — 54 2,26 — — — 2,18 — — 2,12	blunt
13.2	12.4	45 — 46 — 43 2,09 — 2,21 — 2,13	blunt
	11.4	52 — 52 — 60 2,15 — 2,16 — 1,40	blunt
10.9	10.8	28 — 32 — 39 — 48 1,99 — 1,76 — 2,15 — 1,58	blunt
	10.5	39 — 52 — 38 — 40 1,98 — — — 2,15 — 2,12	blunt

Table 4.

Shape and size of muscle fibres from m. gluteus max. of the guinea pig, fixed by boiling.

In m. obliquus abd. intern. a number of muscle fibres with free ends was found. As also observed by *Bardeen* (1903) m. obliquus abd. extern. is on the other hand divided by tendinous septa in segments 20—40 mm. wide. Between the tendinous septa most of the muscle fibres extend from tendinous septum to tendinous septum, while in the foremost part of the muscle, to which the tendinous inscriptions do not reach, there is a number of muscle fibres with free ends. In m. serratus post. the fibres have alle been found to extend from tendon to tendon.

The diameters of the fibres measured on living muscle fibres

		Thickness of Fibres (in μ)													
		30—40	40—50	50—60	60—70	70—80	80—90	90—100	100—110	110—120	120—130	130—140	140—150	150—160	160—170
		Number													
Guinea pig:															
M. gluteus max.	(single muscle)	5	11	11	3										
- — —	(various muscles)	5	11	16	13	2									
M. obliquus abd. intern.	(— —)	4	4	9	7	6	1	1							
M. serratus post.	(— —)	3	7	4	1	1	1								
Rabbit:															
M. serratus post.	(single muscle)	1	2												
Goat:															
M. serratus ant.	(various muscles)	1	1	10	4										
Cat:															
M. serratus post.	(— —)	2	1	1											
Frog:															
M. semitendinosus	(— —)	6	8	24	19	24	23	21	19	25	16	18	9	5	
(Buchthal & Knappeis 1938)															

Table 5.

Thickness of muscle fibres from various muscles of the guinea pig, various mammalian muscles and frog muscles measured on isolated living muscle fibres at equilibrium length.

at equilibrium length appear from table 5. For the purpose of comparison *Buchthal's & Knappeis'* measurements of the diameter of muscle fibres from the frog's m. semitendinosus (*Buchthal & Knappeis*, 1938) have been reproduced. The materials are of course selected, but may give an approximate impression of the magnitude of the fibre diameters although not the exact mean or correct distribution. The table also shows that there is no essential difference between the diameters of the fibres from different muscles from the same animal or from different mammals, while the fibres from the m. semitendinosus of the frog have a more varied and, on the whole, larger diameter than the fibres from the examined mammalian muscles.

CHAPTER III.

MEASUREMENTS OF THE HEIGHT OF MUSCLE COMPARTMENT AND OF THE RATIO OF ANISOTROPIC TO ISOTROPIC SUBSTANCE AT REST AND DURING CONTRACTION.

When attempting to correlate the structure of the muscle fibre to its function by histological investigations the attention should primarily be directed to the cross striation of the fibres. Changes in the anisotropic and isotropic layers are especially characteristic features of the contraction process. The technique developed has made it possible to perform a quantitative determination of the variations of the height of the A- and I-layers which occur during stretch and relaxation as well as during contraction, an analysis which may also throw light on the mechanical properties of the A- and I-layers.

Focusing.

When using unstained preparations for histological investigations of muscle fibres the optical image will vary according to the adjustment of the tube of the microscope on account of the structure of the muscle fibre with alternate narrow cross striae of different refractive indices. This fact is the most important reason for the diverging views which have been held with regard to the cross striation of muscles, both as to the number of cross striae and the magnitude of the latter in the individual compartment as well as to the structural changes during contraction.

Already *Engelmann* (1873) emphasized the importance of focusing; i. e. to obtain clear microscopical images the orientation of the individual transverse discs must be plane-parallel and perpendicular to the focal plane, the illumination must

be centered, with the rays perpendicular to the fibres, the microscope tube must be focused on the centre of the muscle fibres, i. e. it must be adjusted in such a way that the fibres show sharp outlines and appear as wide as possible. The thicker the preparation and the narrower the cross striation the greater the possibility of optical sources of error. *Exner* (1887) tried to explain the diffraction phenomena, "Beugungsbilder", theoretically, and the topic has lately been discussed by *W. J. Schmidt* (1937, pp. 154—158). In case of high focusing the refraction line (Becke's line) separating the two media of different refractive indices will fall within the field of the medium with the highest refractive index; in case of deep focusing it will fall within the medium with lowest refractive index. For muscle fibres this means that while in case of deep focusing the anisotropic substance appears dark the opposite may be the case with high focusing — a fact which was already demonstrated by *Rollett* (1891). *W. J. Schmidt* mentions other optical sources of error: on account of the grating structure of the muscle fibres afocal interference phenomena may occur, and the birefringence of the myofibrils may cause superimposed images corresponding to the two refractive indices. According to *W. J. Schmidt* the optical images of muscle fibres examined between crossed nicols should, on the other hand, be reliable.

When examining muscle fibres in ordinary light deep adjustment of the tube of the microscope will give correct optical images, and such focusing has consequently been used for the following experiments — i. e. a focusing which makes the anisotropic substance appear dark while the isotropic appears light. Only in a few cases in which the optical conditions have been especially unfavourable has it been impossible to obtain correct focusing. Then the A-substance has appeared light, the I-substance dark, and the ratio A:I has been measured "reversed". Such erroneous registrations are, however, quite easy to recognize as in that case the assumed A-substance is found to be smaller than the I-substance, while all other measurements have shown that the A-substance amounts to more than 50 per cent of the total height of the compartment both in resting and contracted fibres.

Otherwise, variations in the adjustment of the tube of the microscope will cause no errors in the quantitative determination of the ratio A:I, as shown in table 6. Microphotographs

Muscle	Preparation	Photo No.	Magnification of Photo	Illumination	Adjustment of Tube	A (in μ)	I (in μ)	A + I (in μ)	$\frac{A}{A+I} \times 100$
m. gluteus max.	82 I	1188 a	121	ord.	high	1.96	1.11	3.07	63.8
		1190 a	121	ord.	medium	1.91	1.13	3.04	62.8
		1191 a	121	ord.	deep	1.97	1.10	3.07	64.2
m. gluteus max.	87 IV	1439 a	236	ord.	high	1.71	1.02	2.73	62.7
		1442 a	236	ord.	deep	1.72	1.02	2.74	62.8
m. gluteus max.	95 I	1524 a	121	pol.	high.	1.74	1.00	2.74	63.5
		1526 a	121	pol.	medium	1.73	0.99	2.72	63.6
		1528 a	121	pol.	deep	1.77	0.98	2.75	64.3
m. gluteus max.	98 II	1602 a	236	pol.	high	1.48	0.93	2.41	61.4
		1603 a	236	pol.	medium	1.48	0.90	2.38	62.2
		1604 a	236	pol.	deep	1.43	0.87	2.30	62.2

Table 6.

A, I, A + I and $(A/A + I) \times 100$ measured on muscle fibres with different adjustments of the microscope tube.

of the same muscle fibre exhibit the same ratio A:I even if they are made with different adjustments of the tube — and this applies whether the fibres are examined in ordinary light or between crossed nicols. Muscle fibres showing atypical or “complex” cross striation (*Hürthle*, 1909) have never been used, as such appearances may also be caused by spurious optical images or by damaged preparations.

Sharpness of images.

The cross striation is only measured when it appears sharply outlined. Measurements performed on blurred pictures would first and foremost result in inaccurate determinations of the ratio A:I, as it is impossible to know at what point of the transition zone the cobweb should be placed.

Blurred pictures may furthermore be due to relative displacement of the myofibrils, movements of the preparation during exposure or, of course, defective focusing. Displacement of fibrils results in transition zones where the light passes both isotropic and anisotropic layers (conf. *Engelmann's* requirement: plane-parallel transverse discs perpendicular to the focal plane). The transition zone will become wider the more reduced the illumination used and the smaller the depth of focus of the objective employed. In muscle fibres in which the cross striae have appeared curved or fairly oblique in relation to the cross diameter of the fibres a displacement of the fibrils may likewise be the explanation, for which reason such preparations have also been discarded.

Accuracy of measurements. Dispersion.)*

The coefficient of variation (the standard deviation in per cent of the mean value) of the measurements of the ten compartments, which have always been determined for each fibre,

*) Dispersion = standard deviation $\left(\sigma = \sqrt{\frac{\sum (x - \bar{x})^2}{n}}\right)$. For materials below 50 σ has been calculated according to the formula

$\left(\sigma = \sqrt{\frac{\sum (x - \bar{x})^2}{n - 1}}\right)$. The standard error of the mean (ϵ) is

equal to $\frac{\sigma}{\sqrt{n}}$. Where nothing else is stated $\pm 2.58 \epsilon$ is used

(precision P = 1 per cent). When dealing with materials con-

is independent of the magnification (table 7), a fact which indicates that the dispersion is not due to the technique of the measurements, but predominantly expresses an actual variation. The coefficient of variation is of the same magnitude for fibres examined in ordinary light and for fibres examined between crossed nicols. Tables 7—9 show that the coefficient of variation is usually smallest for the mean value of A expressed in per cent of the total height of the compartment $((A/A + I) \times 100)$ of the resting fibres. This may be taken as further proof that the dispersion found is indicative of an actual variation rather than of an inaccurate technique; the height of the different compartments of the same fibre may very well vary somewhat as the tension need not be the same throughout the fibre, (connective tissue, adhesion to slides, etc. may exert a certain influence), but this does not result in essential variations in the ratio $A:I$ of the various compartments in the fibre, the $A:I$ ratio being only slightly altered when the fibres are stretched. .

These phenomena may be demonstrated by measuring 120 successive compartments of a single fibre as shown in fig. 12. In this figure each point represents the mean value of ten successive compartments, while the mean of all the measurements is represented by a straight line; the hatched area denotes the dispersion of the individual results in relation to the total mean value, calculated on the basis of groups of measurements consisting of ten each. The coefficient of variation of the height of compartment is in this case 5.2, as against the coefficient of variation of $(A/A + I) \times 100$ which is 2.5.

Measurements are only performed on fibres within reversible degrees of elongation. In highly stretched fibres a relative displacement of myofibrils is often observed without the function of the fibres being altered, a cross striation imitating a helicoidal structure may f. inst. be seen as mentioned on p. 25. Such fibril displacements has no compromising influence on the measurements of stretched fibres. As all experiments have been discarded in which the outlines

sisting of less than 30 individual observations (n), the altered t distribution should be allowed for, and the standard error of the mean multiplied by a figure (t) which, with the existing degrees of freedom ($n - 1$) corresponds to the 1 per cent limit ($p = 1$ per cent (*Kemp*, 1942, pp. 85—96).

Muscle	Preparation	Photo No.	Objective	Eyepiece	Camera	Magnification of Photo	Mean Value			Coefficient of Variation	
							A (ln μ)	I (ln μ)	A + I (ln μ)	$100 \times \frac{(A+I)}{A}$	$100 \times \frac{(I+A)}{A}$
m. serrat post.	57 I	690 α	40 X	10 X	L	121 X	1.46	0.83	2.29	63.7	2.7
		692 α	70 X	10 X	L	236 X	1.45	0.82	2.27	63.9	1.9
m. glut max.	92 II	1492 α	40 X	10 X	L	121 X	1.43	0.86	2.29	62.4	3.0
		1495 α	70 X	10 X	L	236 X	1.47	0.88	2.35	62.5	2.8
m. glut max.	105 I	1691 β	40 X	10 X	L	121 X	1.49	0.89	2.38	62.6	4.4
		1694 β	40 X	10 X	F	202 X	1.46	0.91	2.37	61.6	3.2
m. glut max.	95 II	1533 α	40 X	10 X	L	121 X	1.54	0.88	2.42	63.6	4.2
		1530 α	70 X	10 X	L	236 X	1.51	0.91	2.42	62.4	2.6
m. glut max.	74 II	1021 α	40 X	10 X	L	121 X	1.47	0.89	2.36	62.3	3.1
		1022 α	70 X	10 X	L	236 X	1.46	0.86	2.32	62.9	3.0
m. glut max.	73 I	980 α	40 X	10 X	L	121 X	1.78	0.98	2.76	64.5	3.4
		979 α	70 X	10 X	L	236 X	1.73	0.95	2.68	64.5	2.6

Table 7.

A, I, A + I and $(A/A + I) \times 100$ measured on the same muscle fibre with different magnifications. Calculation of coefficient of variation.

Muscle	Preparation	Photo No.	Magnification of Photo	Mean Value			Coefficient of Variation		
				A (in μ)	I (in μ)	A + I (in μ)	A	A + I	$100 \times \frac{(I + A)}{A}$
m. glut. max.	70 II	927 α 928 α 934 α	121 \times 121 \times 121 \times	1.20 1.39 2.08	0.73 0.82 1.04	1.93 2.21 3.12	2.4 4.1 3.8	2.0 2.8 2.8	3.1 3.5 2.0
m. serrat. post.	54 II	643 α 645 α 647 α	202 \times 202 \times 202 \times	1.40 2.49 1.52	0.84 1.19 0.88	2.24 3.68 2.40	2.6 2.6 3.4	1.8 1.7 3.3	2.2 1.5 1.3
m. serrat. post.	50 IV	522 β 526 γ	202 \times 202 \times	1.47 1.98	0.90 1.04	2.37 3.02	2.4 3.0	2.0 2.1	1.6 1.6
m. serrat. post.	52 IV	592 γ 598 γ	202 \times 202 \times	1.58 2.42	0.90 1.19	2.48 3.61	2.5 2.5	1.1 2.3	1.9 1.8
m. serrat. post.	51 II	554 δ 557 γ	202 \times 202 \times	1.97 1.27	1.04 0.76	3.01 2.03	2.2 2.8	2.6 1.8	1.4 1.7

Table 8.

A, I, A + I and $(A/A + I) \times 100$ measured on the same muscle fibre at different degrees of stretch. Calculation of coefficient of variation.

Muscle	Preparation	Photo No.	Magnification of Photo	Mean Value			Coefficient of Variation		
				λ (in μ)	I (in μ)	$\lambda + I$ (in μ)	λ	$\lambda + I$	$\frac{(\lambda + I)}{\lambda} \times 100$
m. glut. max.	133 VII	2020 α	121 \times	1.12	0.70	1.82	3.2	3.0	2.3
		2021 α	121 \times	1.03	0.96	1.99	2.4	2.2	1.3
m. glut. max.	132 I	1968 β	121 \times	1.40	0.87	2.27	3.0	1.6	2.8
		1969 δ	121 \times	1.02	0.91	1.93	3.4	2.7	2.7
m. glut. max.	135 I	2125 β	121 \times	1.37	0.92	2.29	2.2	1.8	1.2
		2128 β	121 \times	1.20	1.10	2.30	3.9	2.7	1.9
m. serrat. post.	52 II	582 γ	202 \times	1.51	1.30	2.81	2.9	2.1	1.5
		584 γ	202 \times	1.76	1.01	2.77	2.8	1.9	1.8
m. glut. max.	106 II	1710 α	202 \times	1.86	1.04	2.90	2.7	2.4	1.9
		1711 α	202 \times	1.57	1.30	2.87	3.4	2.7	2.2
m. serrat. post.	50 IV	526 β	202 \times	1.98	1.04	3.02	3.0	2.1	1.6
		528 β	202 \times	1.52	1.34	2.86	3.8	2.8	2.3

Table 9.

λ , I , $\lambda + I$ and $(\lambda/I + I) \times 100$ measured on the same muscle fibre during rest and during contraction. Calculation of coefficient of variation.

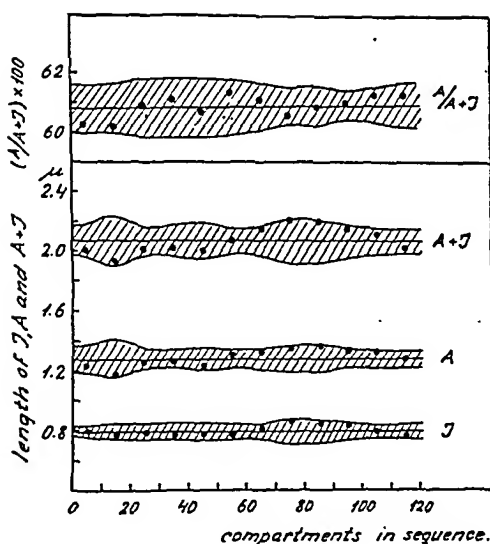


Fig. 12.

The variation of A, I, A + I and $(A/A + I) \times 100$ in a single muscle fibre. Measurements of 120 successive muscle compartments.

Preparation: m. gluteus max. at length 93.

Points plotted: mean values of ten muscle compartments.

Hatched area: dispersion of individual values from the mean value; the dispersion calculated for groups consisting of ten muscle compartments (see text p. 88).

Abscissa: number of successive muscle compartments.

Ordinate: (top) $(A/A + I) \times 100$.

(bottom) length of A + I, A and I in μ .

of the cross striation are blurred, the dispersion of the results remains the same whether the fibres are examined at equilibrium length or in a stretched state (table 8). The experiments likewise show the same dispersion for resting and for contracted fibres (table 9), a uniform and sharply outlined cross striation having also been considered prerequisite when measuring contracted fibres.

As a check on the results numerous fibres are measured two to three times at the same degree of stretch without the result of the first measurement being known when the following were made. Such check measurements are performed on different photographs of the same fibre, photographed with different as well as with the same optical system, see tables 10 and 11. The measurements are, when rendered possible by some distinguishing mark on the fibre, performed on the same part of the fibre in the different photographs. It should be noted that the ratio A : I is the same for the different

Preparation	Photo No.	Magnification of Photo	A (in μ)	I (in μ)	A + I (in μ)	$\frac{A}{(A + I)} \times 100$
70 II	925 α	121 \times	1.28	0.71	1.99	64.3
	927 α	121 \times	1.20	0.73	1.93	62.2
67 I	852 β	121 \times	1.29	0.76	2.05	62.9
	853 β	121 \times	1.30	0.76	2.06	63.1
	854 β	121 \times	1.29	0.74	2.03	63.5
67 I	858 γ	121 \times	1.27	0.74	2.01	63.2
	864 γ	121 \times	1.30	0.74	2.04	63.7
67 I	858 δ	121 \times	1.23	0.71	1.94	63.4
	864 δ	121 \times	1.37	0.83	2.20	62.3
89 III	1472 β	121 \times	1.30	0.79	2.09	62.2
	1473 β	121 \times	1.33	0.79	2.12	62.7
91 II	1480 β	121 \times	1.43	0.88	2.31	61.9
	1481 β	121 \times	1.47	0.87	2.34	62.8
69 II	903 β	121 \times	1.51	0.82	2.33	64.8
	904 β	121 \times	1.52	0.82	2.34	65.0
97 IV	1578 γ	121 \times	1.43	0.90	2.33	61.4
	1581 γ	121 \times	1.47	0.89	2.36	62.3
98 II	1602 β	236 \times	1.48	0.93	2.41	61.4
	1603 β	236 \times	1.48	0.90	2.38	62.2
	1604 β	236 \times	1.43	0.87	2.30	62.2
102 I	1637 γ	236 \times	1.48	0.86	2.34	63.2
	1640 γ	236 \times	1.44	0.87	2.31	63.3
105 I	1691 β	121 \times	1.49	0.89	2.38	62.6
	1694 β	121 \times	1.46	0.91	2.37	61.6
100 I	1615 γ	236 \times	1.50	0.91	2.41	62.2
	1616 γ	236 \times	1.53	0.91	2.44	62.7
96 III	1559 δ	121 \times	1.58	0.93	2.51	63.0
	1561 δ	121 \times	1.54	0.91	2.45	62.8
98 II	1598 γ	121 \times	1.62	0.91	2.53	64.0
	1599 β	121 \times	1.55	0.92	2.47	62.7
96 II	1548 γ	121 \times	1.60	0.95	2.55	62.7
	1549 γ	121 \times	1.59	0.95	2.54	62.6
97 IV	1582 β	121 \times	1.62	0.95	2.57	63.0
	1583 β	121 \times	1.65	0.96	2.61	63.2
87 IV	1439 α	236 \times	1.71	1.02	2.73	62.7
	1442 α	236 \times	1.72	1.02	2.74	62.8
95 I	1524 α	121 \times	1.74	1.00	2.74	63.5
	1526 α	121 \times	1.73	0.99	2.72	63.6
	1528 α	121 \times	1.77	0.98	2.75	64.3

(Table 10 cont.)

Preparation	Photo No.	Magnification of Photo	A (in μ)	I (in μ)	A + I (in μ)	$\frac{A}{A + I} \times 100$
98 I	1585 γ	121 \times	1.80	1.00	2.80	64.3
	1586 γ	121 \times	1.74	1.01	2.75	63.3
98 II	1590 γ	121 \times	1.86	0.98	2.84	65.5
	1593 γ	121 \times	1.73	0.99	2.72	63.6
92 I	1485 β	121 \times	1.75	1.04	2.79	62.7
	1488 β	121 \times	1.64	0.99	2.63	62.4
89 II	1468 β	236 \times	1.83	1.02	2.85	64.2
	1470 β	236 \times	1.84	1.01	2.85	64.5
89 I	1456 γ	121 \times	2.04	1.17	3.21	63.6
	1457 γ	121 \times	2.01	1.12	3.13	64.2
	1458 γ	121 \times	2.02	1.12	2.14	64.3
69 II	911 α	121 \times	2.25	1.12	3.37	66.8
	912 α	121 \times	2.28	1.04	3.32	68.7
72 I	967 γ	121 \times	2.15	1.20	3.35	64.2
	971 γ	121 \times	2.17	1.21	3.38	64.2

Table 10.

Control experiments. Muscle fibres from m. gluteus max. measured 2—3 times on different microphotographs, photographed with the same optical system.

Preparation	Photo No.	Magnification of Photo	A (in μ)	I (in μ)	A + I (in μ)	$\frac{A}{A + I} \times 100$
92 II	1492 α	121 \times	1.43	0.86	2.29	62.4
	1495 α	236 \times	1.47	0.88	2.35	62.5
74 II	1021 α	121 \times	1.47	0.89	2.36	62.3
	1022 α	236 \times	1.46	0.86	2.32	62.9
102 I	1633 δ	121 \times	1.48	0.91	2.39	61.9
	1637 γ	236 \times	1.46	0.87	2.33	62.7
	1640 γ					
74 II	1026 α	121 \times	1.49	0.94	2.43	61.3
	1025 α	236 \times	1.50	0.87	2.37	63.3
73 I	976 α	121 \times	1.54	0.93	2.47	62.3
	977 α	236 \times	1.47	0.89	2.36	62.3
95 II	1533 α	121 \times	1.54	0.88	2.42	63.6
	1530 α	236 \times	1.51	0.91	2.42	62.4

(Table 11 cont.)

Preparation	Photo No.	Magnification of Photo	A (in μ)	I (in μ)	A + I (in μ)	$\frac{A}{(A+I)} \times 100$
72 I	960 β	121 \times	1.55	0.98	2.53	61.3
	962 β	236 \times	1.50	0.92	2.42	62.0
98 II	1598 γ	121 \times	1.59	0.92	2.51	63.3
	1599 β					
	1601 β	236 \times	1.50	0.93	2.43	61.7
73 I	991 α	121 \times	1.66	1.01	2.67	62.2
	993 β	236 \times	1.58	0.94	2.52	62.7
102 II	1645 γ	121 \times	1.73	0.94	2.67	64.8
	1650 β	236 \times	1.69	0.91	2.60	65.0
73 II	994 α	121 \times	1.76	0.93	2.69	65.4
	995 α	236 \times	1.78	0.94	2.72	65.4
73 I	980 β	121 \times	1.78	0.98	2.76	64.5
	979 β	236 \times	1.73	0.95	2.68	64.5
98 I	1585 γ	121 \times	1.77	1.01	2.78	63.6
	1586 γ					
	1589 β	236 \times	1.70	0.96	2.66	63.9
98 II	1590 γ	121 \times	1.80	0.99	2.79	64.5
	1593 γ					
	1596 β	236 \times	1.75	0.98	2.68	63.4
92 I	1485 β	121 \times	1.70	1.02	2.72	62.5
	1488 β					
	1490 β	236 \times	1.83	1.03	2.86	64.0
89 II	1465 β	121 \times	1.90	1.03	2.93	64.8
	1468 β	236 \times	1.84	1.02	2.86	64.3
	1470 β					
89 I	1456 γ	121 \times	2.02	1.14	3.16	63.9
	1457 γ					
	1458 γ	236 \times	1.99	1.11	3.10	64.2
	1459 α					
82 II	1218 γ	121 \times	2.28	1.15	3.43	66.5
	1213 β	236 \times	2.24	1.14	3.38	66.3

Table 11.

Control experiments. Muscle fibres from m. gluteus max. measured 2—3 times on different microphotographs, photographed with different magnifications.

measurements of the same fibre. The absolute figures (height of compartment, height of A and I) may, however, differ, as stretch or relaxation of the fibres may occur between one exposure and the next. Consistency between two series of stretch experiments on fibres from m. gluteus max. — ex-

aminated in ordinary light and between crossed nicols respectively (table 14, fig. 15) — can also be considered a proof of the accuracy of the measurements (conf. W. J. Schmidt, 1937, p. 156).

Height of Compartment at Equilibrium Length.

On muscle fibres from *m. semitendinosus* of the frog *Buchthal, Knappeis & Lindhard* (1936) have shown that the height of compartment at equilibrium length is a reproducible value, fairly constant for different fibres from the same muscle, also if from different frogs, the variations amounting to abt. 10 per cent (*Buchthal*, 1942, p. 49). However, the muscle fibres are not perfectly elastic (see chapter V. A), an irreversible plastic elongation setting in when they are stretched more than 40—50 per cent, a fact which has been demonstrated e. g. by *Asmussen* (1936), but this plastic elongation is only small and in the present material it never exceeds 10 per cent.

In practice it is more difficult to determine the equilibrium length in mammalian muscles, as in most cases the material has to be small bundles and not individual fibres, which may be used in case of frog muscles. The difficulties met with are greater in case of a small fibre bundle than in case of a single fibre when trying to ascertain exactly when the bundle or fibre is just straightened out, and the individual fibres of a small bundle are not always stretched to the same degree. Table 12 shows the variation of the heights of compartments

Muscle	Preparation	Height of Compartment in the Different Fibres (in μ)			
<i>m. obliquus abd. intern.</i>	58 II	2.16	2.13	1.97	1.91
<i>m. gluteus max.</i>	67 I	1.99	2.04	2.03	2.07
<i>m. obliquus abd. intern.</i>	59 III	2.11	2.16	2.20	2.08
<i>m. serratus post.</i>	56 II	2.35	2.29	2.30	2.37
<i>m. gluteus max.</i>	96 III	2.48	2.59	2.40	
<i>m. serratus post.</i>	57 I	2.59	2.74	2.64	
<i>m. obliquus abd. intern.</i>	73 III	3.25	3.20	3.05	3.27
<i>m. serratus post.</i>	57 I	3.60	3.69	3.41	3.56

Table 12.

Height of compartment measured in different muscle fibres of the same bundle.

— and consequently of the elongation — of the individual fibres of a bundle. The difference in degree of stretch within the same bundle is due to variations in the dimensions of the fibres which cause a certain tension to produce different elongations of the fibres. Connective tissue or slight twisting of the bundle may furthermore cause uneven stretch. A relatively large material is therefore required to obtain a sufficiently exact determination of the equilibrium length.

	Number	Mean Value (in μ)	Coefficient of Variation	Dispersion (in μ)	Standard Error of the Mean	
					(in μ)	(P = 1 %)
m. gluteus max.	60	2.24	0.042	1.9	0.005	0.014
m. serratus post.	36	2.23	0.134	6.0	0.022	0.058
m. obliquus abd. intern. . (excised)	38	2.23	0.177	7.9	0.029	0.074
m. obliquus abd. intern. . (in situ)	29	2.22	0.182	8.2	0.034	0.093

Table 13.

Mean values and dispersion of the height of compartment of muscle fibres at equilibrium length, measured on various muscles of the guinea pig. In the second column the number of muscle fibres measured is stated.

M. gluteus max. is the muscle preferably used; at equilibrium length its fibres exhibit a height of compartment of $2.24 \pm 0.01 \mu$ (see table 13). This measurement has been performed on 60 different fibres and is consequently the mean value of 600 compartments. In this material the variation between the individual fibres seems to be small; the coefficient of variation (for P = 1 per cent) is approximately 5.

For fibres from m. serratus post. and m. obliquus abd. intern. practically the same mean value as to height of compartment at equilibrium length has been found: 2.23μ . The dispersion is somewhat larger here (see table 13), consequently the experiments do not provide any statistical proof that the height of compartment at equilibrium length is the same for different muscles.

With regard to the m. obliquus abd. intern. the equilibrium length is measured for excised fibres as well as for fibres in situ. Only a small part of the length of the fibres in situ has been isolated — at most $\frac{1}{2}$ — $\frac{3}{4}$ cm. — the circulation and innervation being as far as possible left intact. The intact

innervation manifests itself by the movements of respiration of the isolated muscle fibres which are sometimes met with during the experiments and are due to co-innervation. When this method is used the state of the fibre approaches the natural conditions as much as technical considerations will allow. It was the intention to find out whether muscle fibres in situ behave otherwise than excised muscle fibres, i. a. with the purpose — if possible — of obtaining information with respect to the “tone” of the muscle, if any. The determination of the height of compartment of the muscle fibre at equilibrium length under the two different conditions — in situ and excised — has, however, yielded no exact information as to difference in equilibrium length — the dispersion being too large (table 13). For excised fibres the height of compartment is found to be $2.23 \pm 0.07 \mu$, for fibres in situ $2.22 \pm 0.09 \mu$. Another way is therefore chosen of showing the consistency between excised muscle fibres and fibres in situ — i. e. a comparison between the curves representing the variation of the ratio A:I as a function of stretch of the fibres.

The Ratio A:I at Equilibrium Length and during Stretch of Resting Fibres.

The ratio A:I in the individual compartment is expressed as the height of A in per cent of the total height of compartment ($(A/A + I) \times 100$), as it is the A-substance which changes its equilibrium length during contraction — is shortened — while this is not the case with the I-substance.

At equilibrium length A amounts to about 62 per cent of the total height of the compartment in fibres from m. gluteus max. (table 17). Fig. 13 shows 5 single experiments in which individual fibres are measured at different degrees of stretch. The experiment shows that the ratio A:I differs slightly in the different muscle fibres, while, as previously shown, the ratio A:I in compartments of the same fibre only varies insignificantly. The dispersion is at any rate evidently smaller than the difference between the ratio A:I of fibres at equilibrium length and of stretched fibres. In all experiments it is clearly seen that when the fibres are stretched, A takes up a greater percentage of the height of the compartment — A is stretched relatively more than I; in other words the stiffness

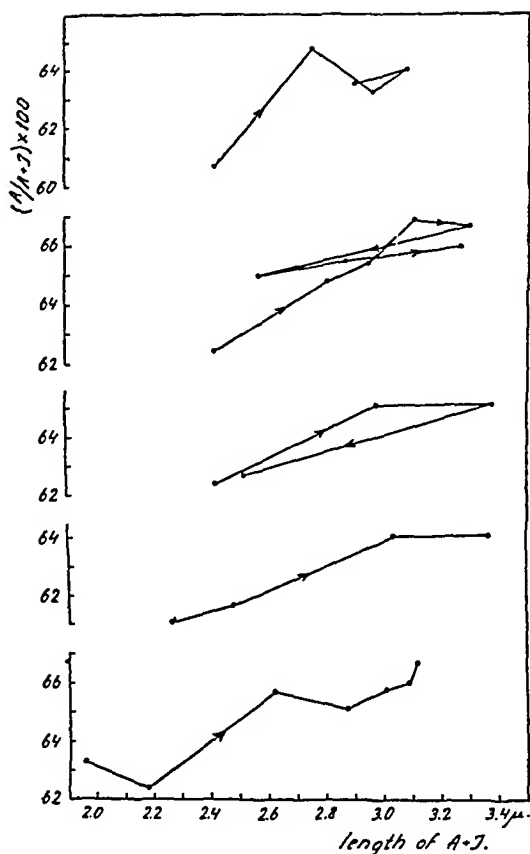


Fig. 13.

The ratio $A:I$ (expressed as $(A/A + I) \times 100$) as a function of the degree of stretch of the muscle fibre (expressed as height of compartment). Experimental series with 5 different muscle fibres from *m. gluteus max.*

The arrows denote the sequence of the experiments.

Abscissa: length of $A + I$ in μ .

Ordinate: $(A/A + I) \times 100$.

of A is less than that of I (conf. Chapter V. D). Figs. 13, 21—22 and 37 also show that the alterations produced by the stretching are reversible.

The fact that A is stretched relatively more than I is most clearly seen when calculating the elongation of A in per cent of the height of the A -substance at equilibrium length and, in the same way, the elongation of I in per cent of the height of I at equilibrium length. The mean values of all measurements made on resting fibres from *m. gluteus max.* are calculated in this manner (table 17, columns 9—10), and fig. 14 shows the corresponding diagrams: the percentage elongation of A and I as a function of the percentage elongation

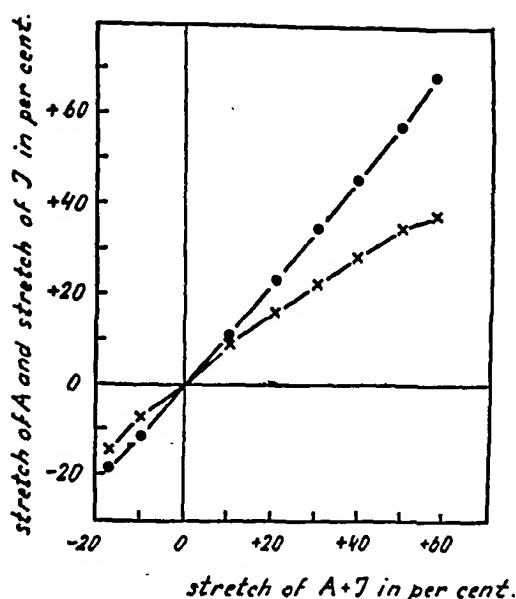


Fig. 14.

Variations in the heights of A- and I-layers as a function of the degree of stretch of the fibres. Mean values from all experiments on muscle fibres from m. gluteus max. (conf. table 17).

Abscissa: elongation (or shortening) of the height of compartment (A + I) in per cent of the height of compartment at equilibrium length.

Ordinate: elongation (or shortening) of A and I in per cent of A and I at equilibrium length.

M. gluteus max. (in ordinary light)		M. gluteus max. (between crossed nicols)	
(in μ) A + I	A/A + I $\times 100$	A + I (in μ)	A/A + I $\times 100$
1.92	61.7	2.23	62.2
2.01	61.7	2.38	62.1
2.12	61.8	2.48	62.5
2.25	62.1	2.58	63.3
2.34	63.1	2.77	63.9
2.42	62.3	(2.94	64.1)
2.48	62.3		
2.60	63.0		
2.74	64.3		
2.87	64.2		
3.00	64.9		
3.19	65.8		
3.41	66.1		

Table 14.

Correlation of degree of stretch (height of compartment) to ratio A : I ((A/A + I) $\times 100$) in muscle fibres from m. gluteus max.

Measurements on muscle fibres photographed in ordinary light and photographed between crossed nicols.

Each figure is the mean value of 10 experiments, i. e. of 100 measurements.

of the compartment. It should be noted that when the fibres are compressed below equilibrium length, the smaller stiffness of the A substance is also disclosed by the fact that A is shortened more than I — the curves intersect at equilibrium length.

In order to ensure that the measurements of the ratio A : I are correct some of the fibres from m. gluteus max. are examined between crossed nicols. In table 14 the two materials — fibres examined in ordinary light and between crossed nicols — are arranged according to increasing degree of stretch to show the relationship of extension — expressed as the absolute value of the height of the compartment — to the ratio A : I — expressed as A in per cent of the total height of compartment.

By means of numerical fitting according to the "method of least squares" the curve of regression has been calculated (*Kemp, 1942*, pp. 135—147). $b_{xy} = \frac{\sum (x - \bar{x})(y - \bar{y})}{(x - \bar{x})^2}$ expresses the calculated regression coefficient of the height of compartment, in the equation represented by x , in proportion to $(A/A + I) \times 100$ which is represented by y . \bar{x} and \bar{y} are mean values of $(A + I)$ and $(A/A + I) \times 100$ respectively, calculated on the basis of the whole material — x and y being the individual values. The regression coefficient of $(A/A + I) \times 100$ in proportion to $(A + I)$ is calculated in a similar manner from the formula $b_{yx} = \frac{\sum (x - \bar{x})(y - \bar{y})}{(y - \bar{y})^2}$.

The lines of regression may now be determined according to the equations $Y - \bar{y} = b_{xy} (X - \bar{x})$ and $X - \bar{x} = b_{yx} (Y - \bar{y})$, it being possible for every value of Y or X to calculate the corresponding values of X or Y .

As the regression is nearly linear this procedure is considered to be justified. The curve plotted in the diagrams shown is the mean curve of the two lines of regression.

From fig. 15 it can be seen that the mean curves for fibres examined in polarized light and in ordinary light have practically the same inclination, and the magnitude of $(A/A + I) \times 100$ is also the same in these two materials. The ratio A : I thus seems to be correctly measured and the two materials may be lumped together and considered as a whole as done in tables 16 and 17 and fig. 17 and 18.

The results of the two series of experiments on fibres from m. obliquus abd. intern. — excised and in situ — are treated in the same manner as the m. gluteus max. experiments (table

15 and fig. 16). As it appears from fig. 16 there is no difference between the ratio A:I of fibres in situ and that of excised fibres, and stretch has the same effect on the fibres irrespective of the two different sets of experimental conditions. Consequently it may also be justified to consider these two materials as a whole.

The ratio A:I and its change in case of stretch are practically identical in muscle fibres from m. obliquus abd. intern. and m. serratus post. (table 16 and fig. 17), but fibres from

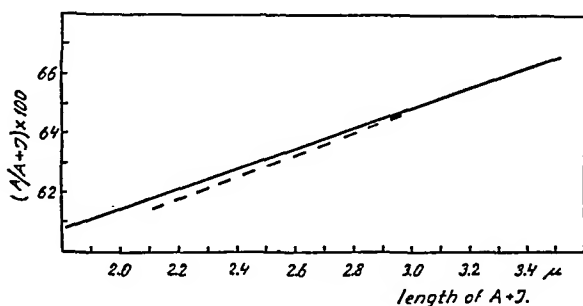


Fig. 15.

Correlation lines of the ratio A:I as a function of the degree of stretch of fibres from m. gluteus max., examined in ordinary light (—) and between crossed nicols (---).

The correlation is calculated according to the values of table 14 (see text p. 101).
Abscissa: (A + I) in μ .

Ordinate: $(A/A + I) \times 100$.

M. obliquus abd. intern. (excised)		M. obliquus abd. intern. (in situ)	
A + I (in μ)	A/A + I $\times 100$	A + I (in μ)	A/A + I $\times 100$
1.95	62.1	1.99	62.6
2.04	62.8	2.11	62.9
2.10	62.3	2.37	61.8
2.17	63.0	2.64	64.0
2.22	62.8	3.04	64.1
2.36	63.6	3.88	66.3
2.43	63.1		
2.56	63.9		
2.72	63.2		
3.08	65.0		
3.52	65.7		
(3.97)	(65.2)		

Table 15.

Correlation of degree of stretch (height of compartment) to ratio A:I ($(A/A + I) \times 100$) in muscle fibres from m. obliquus abd. intern. measured on excised fibres and fibres in situ.

Each figure is the mean value of 4 experiments, i. e. of 40 measurements.

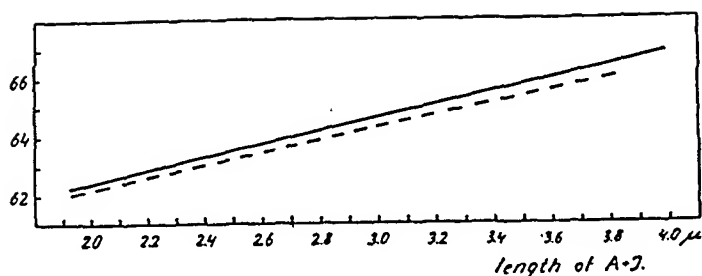


Fig. 16.

Correlation lines of the ratio A : I as a function of the degree of stretch of fibres from m. obliquus abd. intern., excised (—) and in situ (---).

The correlation is calculated according to the values of table 15.

Abscissa: (A + I) in μ .

Ordinate: (A/A + I) \times 100.

Guinea pig						Goat	
M. glut. max.		M. serrat. post.		M. obliq abd. intern.		M. serrat. ant.	
A + I (in μ)	A/A + I \times 100	A + I (in μ)	A/A + I \times 100	A + I (in μ)	A/A + I \times 100	A + I (in μ)	A/A + I \times 100
1.92	61.7	1.94	62.5	1.98	62.4	2.09	62.4
2.01	61.7	2.15	62.5	2.09	62.6	2.31	62.4
2.11	61.8	2.28	63.1	2.22	62.8	2.40	62.2
2.21	62.6	2.41	62.7	2.40	62.9	2.50	63.0
2.28	62.0	2.64	63.0	2.61	63.5	2.71	63.6
2.34	62.9	2.78	63.9	3.04	64.8	2.79	63.8
2.38	62.7	2.88	64.1	3.78	65.9	2.89	64.6
2.42	61.8	3.05	65.0			2.99	64.6
2.46	62.5	3.29	65.3			3.30	65.0
2.49	62.5	3.58	66.1			(3.76	66.0)
2.55	63.1	(3.97	66.2)				
2.63	63.0						
2.72	63.9						
2.78	64.2						
2.88	64.3						
2.97	64.7						
3.14	65.5						
3.36	65.9						
(3.52	66.7)						

Table 16.

Correlation of degree of stretch (height of compartment) to ratio A : I ((A/A + I) \times 100) in muscle fibres from m. gluteus max., m. serratus post. and m. obliquus abd. intern. of guinea pig and m. serratus ant. of goat.

Each figure is the mean value of 10 experiments, i. e. of 100 measurements; with regard to m. serratus ant. of goat the figures are mean values of 4 experiments only, i. e. of 40 measurements.

m. gluteus max. behave somewhat differently: here the A-substance amounts to a somewhat smaller percentage of the total height of compartment at equilibrium length. 62.3 per cent for m. gluteus max., 63.0 per cent for m. serratus post. and 62.9 per cent for m. obliquus abd. intern. On the other hand, when comparing the extensibility of A during stretch with that of I the A-substance is found to be more extensible in the m. gluteus max. than in the other muscles, $(A/A + I) \times 100$ increases to 66.0 per cent at length 150 in fibres from m. gluteus max. while the corresponding value for m. obliquus

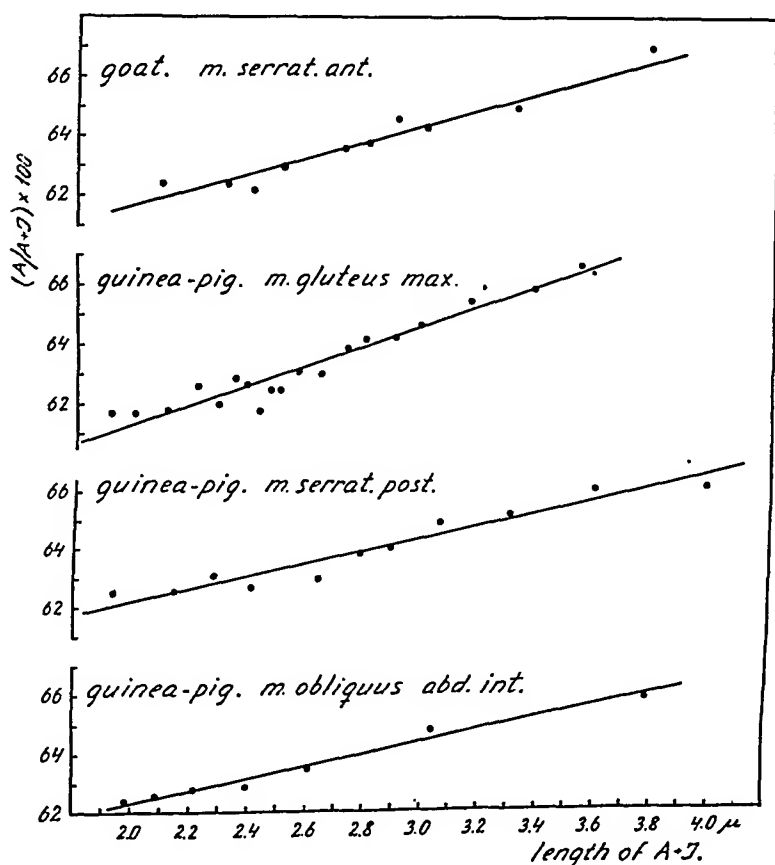


Fig. 17.

Correlation lines of the ratio $A : I$ as a function of the degree of stretch on the basis of all the experiments on fibres from m. serratus ant. of goat, m. gluteus max., m. serratus post. and m. obliquus abd. intern. of guinea pig. The correlation is calculated according to the values of table 16. Every point plotted thus denotes the mean value of ten experiments = 100 measurements; with regard to the m. serratus ant. of goat the mean value of four experiments only (= 40 measurements).

Abscissa: length of $A + I$ in μ .

Ordinate: $(A/A + I) \times 100$.

abd. intern. and m. serratus post. are 65.2 per cent and 65.3 per cent respectively.

All the experiments performed on resting fibres from m. gluteus max. are arranged according to the degree of stretch of the fibres and divided in groups for every 10 per cent of stretch. The mean values for these groups are calculated in table 17 and fig. 18. This division in groups has the drawback

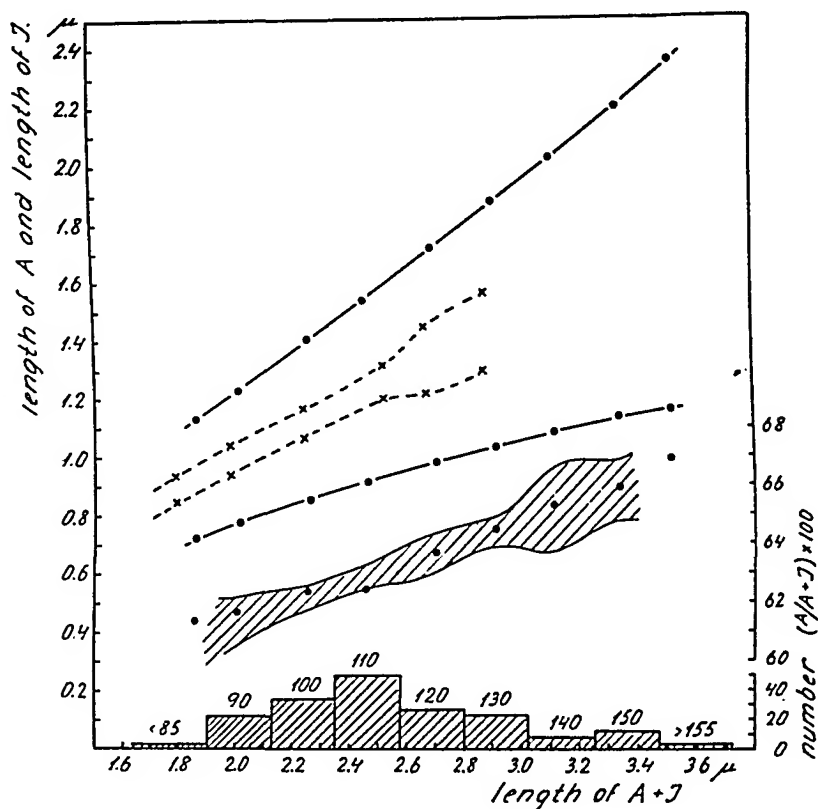


Fig. 18.

Length of A- and I-substance during rest (•) and during contraction (×) as a function of the degree of stretch of the fibres, determined on muscle fibres from m. gluteus max. Whole material.

The height of the hatched columns denotes the number of experiments by which the resting values have been determined within each group (length 85—95, 95—105, etc.). The hatched area represents the standard error of the mean (for $p = 1$ per cent) of the calculated correlation curve between $(A/A+I) \times 100$ at rest and the degree of stretch of the fibres (conf. fig. 17). The standard error of the mean is calculated for each group separately.

Abscissa: length of $A + I$ in μ .

The figures above the hatched columns denote length of fibre (equilibrium length = 100).

Ordinate: (to the left) length of A (two top curves) and I (two bottom curves) at rest (—) and during contraction (— — — —) in μ .

(to the right) $(A/A + I) \times 100$.

Number of experiments.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Rest										Contraction						
Length of Fibre (Equilibrium Length = 100)	Height of Comp- artment in μ	Number	A (in μ)	I (in μ)	(A + I) (in μ)	$\frac{A}{A + I} \times 100$	Degree of Stretch (Equilibrium Length = 100)	Percentage of A Elongation or Shortening of A	Percentage of I Elongation or Shortening of I	Number	A (in μ)	I (in μ)	(A + I) (in μ)	$\frac{A}{A + I} \times 100$	Percentage of A Shortening of A	Percentage of I Elongation of I
85	(—1.90 μ)	3	1.14	0.72	1.86	61.4	83.0	—18.5	—14.5	7	0.94	0.85	1.79	52.5	12.7	19.2
90	(1.91—2.13 μ)	23	1.23	0.78	2.01	61.7	89.7	—12.0	—7.0	19	1.04	0.94	1.98	52.4	13.8	21.5
100	(2.14—2.35 μ)	34	1.41	0.85	2.26	62.4										
110	(2.36—2.58 μ)	50	1.54	0.92	2.46	62.5	109.8	+10.0	+9.5	8	1.17	1.07	2.24	52.3	14.2	22.4
120	(2.59—2.80 μ)	27	1.72	0.98	2.70	63.7	120.5	23.0	16.5	2	1.32	1.20	2.52	52.6	14.3	22.7
130	(2.81—3.02 μ)	23	1.88	1.03	2.91	64.5	129.9	34.5	22.5	3	1.45	1.22	2.67	54.2	12.0	19.6
140	(3.03—3.25 μ)	8	2.03	1.08	3.11	65.3	138.8	45.0	28.5	1	1.57	1.30	2.87	54.7	14.5	25.8
150	(3.26—3.47 μ)	12	2.20	1.14	3.34	65.9	149.1	57.0	35.5							
155	(3.48— μ)	3	2.36	1.16	3.52	66.9	157.4	68.5	38.0							

Table 17.

Mean values of all experiments on muscle fibres from m. gluteus max.

The material is divided according to the degree of stretch of the fibres (length 85—95, 95—105 etc.). Columns 3 and 11 state the number of muscle fibres measured within each degree of stretch.

Column 8 states the height of compartment as a function of the mean value of compartment height at equilibrium length (2.24 μ). Equilibrium length = 100.

In columns 9 and 10 the elongation or shortening of A and I is calculated in per cent of the mean value of the length of A and I at equilibrium length (1.40 μ and 0.84 μ respectively).

In columns 16 and 17 the shortening of A and the elongation of I during contraction is stated in per cent of the length of A and I respectively during rest at the same degree of stretch; mean values of the individual figures within each group in table 20 columns 13 and 14.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Length of Fibre (Equilibrium Length = 100)	Height of Com- partment in μ	Rest						Contraction								Percentage of I Shortening of A
		Number	A (in μ)	I (in μ)	(A + I) (in μ)	$\frac{A + I}{A} \times 100$	Degree of Stretch (Equilibrium Length = 100)	Percentage of A Shortening of A	Percentage of I Elongation or Shortening of I	Number	A (in μ)	I (in μ)	(A + I) (in μ)	$\frac{A + I}{A} \times 100$	Percentage of A	Percentage of I
85	(—1.90 μ)	2	1.15	0.70	1.85	62.2	83.0	—18.0	—15.5	1	1.10	0.97	2.07	53.2	13.1	20.6
90	(1.91—2.12 μ)	14	1.25	0.75	2.00	62.5	89.5	—10.5	—	1	1.22	1.05	2.27	53.8		
100	(2.13—2.34 μ)	18	1.43	0.84	2.27	63.1				5	1.37	1.14	2.51	54.4	13.2	22.9
110	(2.35—2.56 μ)	10	1.54	0.92	2.46	62.4	110.5	+ 10.0	+ 11.0	8	1.47	1.24	2.71	54.1	15.0	26.1
120	(2.57—2.79 μ)	15	1.72	0.99	2.71	63.4	121.5	23.0	19.5	6	1.56	1.32	2.88	54.3	14.9	26.6
130	(2.80—3.01 μ)	16	1.86	1.03	2.89	64.3	129.5	33.0	24.0	2	1.73	1.35	3.08	56.2	13.0	23.7
140	(3.02—3.23 μ)	10	2.02	1.10	3.12	64.9	140.0	44.5	32.5							
150	(3.24—3.46 μ)	9	2.20	1.16	3.36	65.5	150.5	57.0	39.5							
160	(3.47—3.68 μ)	8	2.39	1.22	3.61	66.2	162.0	70.5	47.0							
170	(3.69—3.90 μ)	2	2.44	1.28	3.72	65.6	167.0	74.5	54.5							
180	(3.91—4.12 μ)	2	2.62	1.34	3.96	66.1	177.5	87.0	61.5							
185	(4.13— μ)	2	2.83	1.41	4.24	66.8	190.0	102.0	70.0							

Table 18.

Mean values of all experiments on muscle fibres from m. serratus post.

The material is divided according to the degrees of stretch of the fibres (length 85—95, 95—105 etc.).

Columns 3 and 11 state the number of muscle fibres measured within each degree of stretch.

Column 8 states the height of compartment as a function of the mean value of compartment height at equilibrium length (2.23 μ). Equilibrium length = 100.

In columns 9 and 10 the elongation or shortening of A and I is calculated in per cent of the mean value of the length of A and I at equilibrium length (1.40 μ and 0.83 μ respectively).

In columns 16 and 17 the shortening of A and the elongation of I during contraction is stated in per cent of the length of A and I respectively during rest at the same degree of stretch; mean values of the individual figures within each group in table 21 columns 13 and 14.

that, as shown in the diagram, the mean values will represent rather varying numbers of individual experiments. The standard error of the mean of the curve representing the ratio $A:I$ as a function of stretch has been calculated separately for the individual values of each group, and the hatched area of the diagram is calculated in such a way that the probability of the mean curve deviating outside this area is less than 1 per cent.

The results of the investigation on fibres from *m. serratus post.* and *m. obliquus abd. intern.* are arranged in a similar manner (tables 18 and 19 and figs. 19 and 20). Apart from the less pronounced changes in the ratio $A:I$ during stretch of the fibres the shape of the curve is similar to that of the *m. gluteus max.*-experiments. Figs. 21 and 22 show some individual experiments. While unambiguous microscopical images have only been obtained of fibres from *m. gluteus max.* stretched up to length 160 it has been possible to measure fibres from *m. serratus post.* at elongations up to length 190.

1	2	3	4	5	6	7	8	9	10
Length of Fibre (Equilibrium Length = 100)	Height of Com- partment in μ	Number	A (in μ)	I (in μ)	(A + I) (in μ)	$\frac{A}{(A + I)} \times 100$	Degree of Stretch (Equilibrium Length = 100)	Percentage Elongation or Shortening of A	Percentage Elongation or Shortening of I
90	(1.91—2.12 μ)	18	1.26	0.76	2.02	62.5	90.5	— 10.0	— 8.5
100	(2.13—2.34 μ)	13	1.40	0.82	2.22	62.9			
110	(2.35—2.56 μ)	12	1.53	0.90	2.43	63.1	109.0	+ 9.5	+ 8.5
120	(2.57—2.79 μ)	9	1.70	0.97	2.67	63.5	119.5	21.5	17.0
130	(2.80—3.01 μ)	2	1.89	1.03	2.92	64.6	131.0	35.0	24.0
140	(3.02—3.23 μ)	4	2.01	1.09	3.10	64.8	139.0	43.5	31.5
150	(3.24—3.46 μ)	3	2.19	1.14	3.33	65.7	149.0	56.5	37.5
155	(3.47—3.68 μ)	3	2.35	1.23	3.58	65.7	160.5	68.0	48.0

Table 19.

Mean values of all experiments on muscle fibres from *m. obliquus abd. intern.* at rest.

Column 3 states the number of muscle fibres measured within each degree of stretch.

Column 8 states the height of compartment as a function of the mean value of compartment height at equilibrium length (2.23 μ). Equilibrium length = 100.

In columns 9 and 10 the elongation or shortening of A and I is calculated in per cent of the mean value of the length of A and I at equilibrium length (1.40 μ and 0.83 μ respectively).

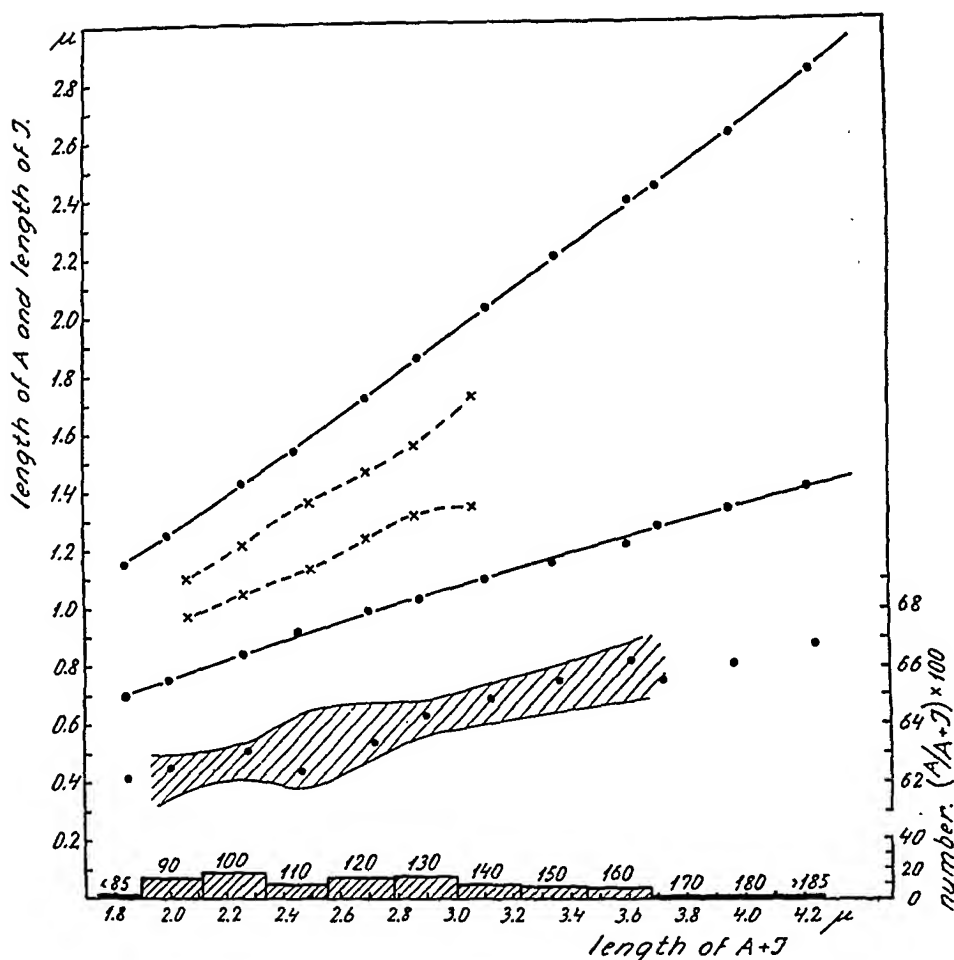


Fig. 19.

Length of A- and I-substance during rest (•) and during contraction (×) as a function of the degree of stretch of the fibres, determined on muscle fibres from *m. serratus post.* Whole material.

The height of the hatched columns denotes the number of experiments by which the resting values have been determined within each group (length 85—95, 95—105, etc.). The hatched area represents the standard error of the mean (for $p=1$ per cent) of the calculated correlation curve between $(A/A+I) \times 100$ at rest and the degree of stretch of the fibres (conf. fig. 17). The standard error of the mean is calculated for each group separately.

Abscissa: length of $A+I$ in μ .

The figures above the hatched columns denote length of fibre (equilibrium length = 100).

Ordinate: (to the left) length of A (two top curves) and I (two bottom curves) at rest (—) and during contraction (---) in μ .

(to the right) $(A/A+I) \times 100$.

Number of experiments.

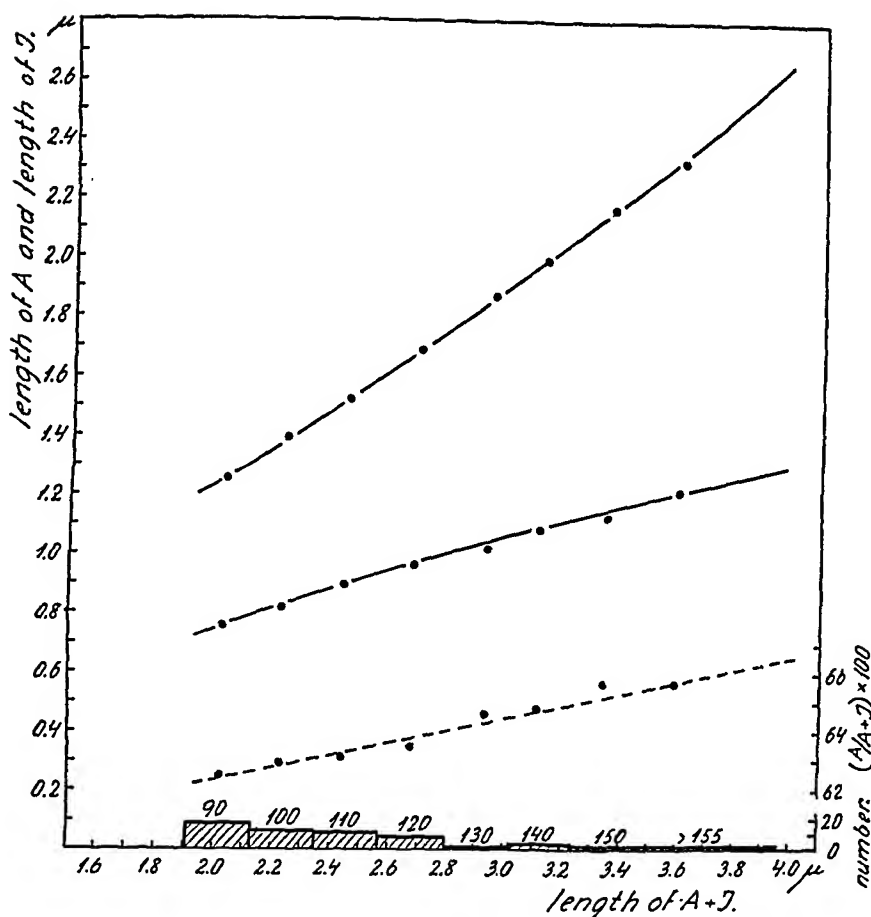


Fig. 20.

Length of A- and I-substance as a function of the degree of stretch of the fibres, determined on resting muscle fibres from m. obliquus abd. intern. Whole material.

The height of the hatched columns denotes the number of experiments according to which the mean figures have been determined within each group (length 85—95, 95—105 etc.).

Abscissa: length of $A + I$ in μ .

The figures above the hatched columns denote length of fibre (equilibrium length = 100).

Ordinate: (to the left) length of A (top curve) and I (bottom curve) in μ .

(to the right) $(A/A + I) \times 100$.

Number of experiments.

Changes in the Ratio A : I during Contraction.

Examinations of mammalian muscle fibres show, like *Buchthal, Knappéis & Lindhard's* experiments on frog fibres (1936), that the A-substance is shortened during contraction, and that a corresponding elongation of the I-substance sets

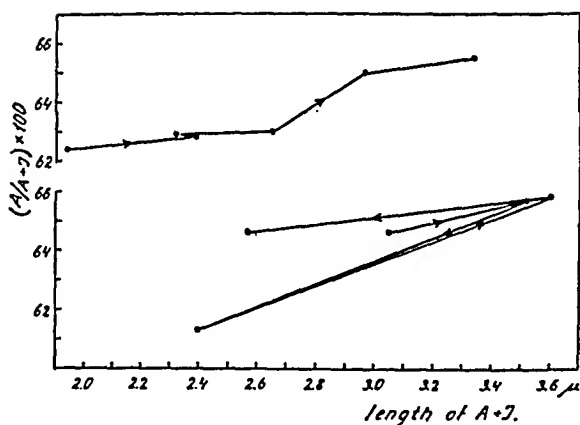


Fig. 21.

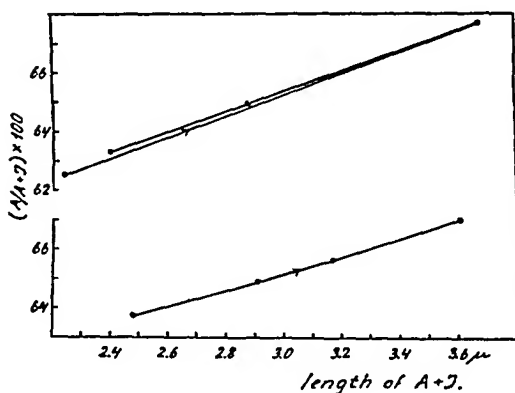


Fig. 22.

Figs. 21—22.

The ratio $A:I$ as a function of the degree of stretch of the fibres. Experimental series with four different muscle fibres from *m. serratus post.* (fig. 21) and from *m. obliquus abd. intern.* (fig. 22).

The arrows denote the sequence of the experiments.

Abscissa: length of $A + I$ in μ .

Ordinate: $(A/A + I) \times 100$.

in when the contraction is isometric. In tables 20 and 21 the results of the individual experiments are arranged in columns according to the extension of the fibres during contraction. It is always the same fibre which is measured during rest and contraction, and the measurements are as far as possible performed on the same part of the fibre. The changes in the ratio $A:I$ registered during contraction have been reversible, several fibres are measured at the same degree of stretch both

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Preparation	Rest						Contraction							
	A (in μ)	I (in μ)	(A + I) (in μ)	$\frac{(A + I)}{A} \times 100$	$\frac{I}{A}$	A (in μ)	I (in μ)	(A + I) (in μ)	$\frac{(A + I)}{A} \times 100$	$\frac{I}{A}$	Degree of Stretch (Equilibrium Length at Rest = 100)	Percentage of A Shortening of A	Percentage of I Elongation of I	
132 I	1.17	0.77	1.94	60.3	1.52	0.91	0.79	1.70	53.5	1.15	75.9	10.1	14.8	
133 IV	1.16	0.72	1.88	61.7	1.61	0.89	0.83	1.72	51.8	1.07	76.8	15.3	24.3	
135 II	1.53	0.92	2.45	62.4	1.66	0.93	0.85	1.78	52.2	1.09	79.5	13.3	20.1	
132 I	1.17	0.77	1.94	60.3	1.52	0.95	0.85	1.80	52.8	1.12	80.4	11.9	17.7	
134 IV	1.21	0.76	1.97	61.4	1.59	0.95	0.88	1.83	51.9	1.08	81.7	14.8	23.0	
133 IX	1.23	0.82	2.05	60.0	1.50	0.96	0.87	1.83	52.5	1.10	81.7	11.3	16.4	
136 I	1.19	0.78	1.97	60.4	1.53	0.98	0.88	1.86	52.7	1.11	83.0	12.2	18.3	
134 IV	1.25	0.80	2.05	61.0	1.56	1.00	0.93	1.93	51.8	1.08	86.2	14.5	22.3	
136 V	1.18	0.74	1.92	61.5	1.60	1.01	0.92	1.93	52.3	1.10	86.2	15.0	23.9	
132 I	1.40	0.87	2.27	61.7	1.61	1.02	0.91	1.93	52.8	1.12	86.2	12.7	19.5	
136 III	1.19	0.73	1.92	62.0	1.63	1.03	0.91	1.94	53.1	1.13	86.6	14.4	23.4	
133 V	1.20	0.77	1.97	60.9	1.56	1.02	0.92	1.94	52.5	1.11	86.6	13.2	20.4	
133 V	1.20	0.83	2.03	59.2	1.45	1.01	0.94	1.95	51.8	1.07	87.0			
133 V	1.22	0.81	2.03	60.1	1.51	1.02	0.93	1.95	52.3	1.10	87.0	11.4	16.4	
134 I	1.49	0.93	2.42	61.6	1.60	1.01	0.94	1.95	51.8	1.07	87.0	13.5	20.2	
135 II	1.20	0.83	2.03	59.2	1.45	1.02	0.94	1.96	52.0	1.09	87.5	13.4	20.0	
133 V	1.20	0.83	2.03	59.2	1.45	1.05	0.91	1.96	53.6	1.15	87.5			
133 V	1.12	0.70	1.82	61.5	1.60	1.04	0.94	1.98	52.5	1.11	88.4	11.2	16.1	
133 VII	1.31	0.84	2.15	60.9	1.56	1.03	0.96	1.99	51.8	1.07	88.8	16.6	27.2	
133 IX						1.03	0.96	1.99	51.8	1.07	88.8	14.3	21.7	

135 I	1.14	0.73	1.87	61.0	1.56	1.06	0.96	2.02	52.5	1.10	90.2	14.6	23.4
136 III	1.19	0.73	1.92	62.0	1.63	1.09	0.93	2.02	54.0	1.17	90.2	13.3	22.0
136 V	1.19	0.73	1.92	62.0	1.63	1.07	0.96	2.03	52.7	1.11	90.6	15.4	25.5
133 VIII	1.24	0.81	2.05	60.5	1.53	1.07	0.97	2.04	52.4	1.10	91.1	13.4	20.5
133 VII	1.21	0.80	2.01	60.2	1.51	1.07	1.00	2.07	51.7	1.07	92.4	14.4	22.0
135 IV	1.36	0.87	2.23	61.0	1.56	1.12	1.01	2.13	52.6	1.11	95.1	13.3	20.6
133 IV	1.18	0.76	1.94	60.8	1.55	1.13	1.01	2.14	52.8	1.12	95.5	12.2	18.3
136 XI	1.55	0.93	2.48	62.5	1.67	1.13	1.04	2.17	52.1	1.09	96.9	15.2	24.1
133 X	1.22	0.73	2.00	61.0	1.56	1.14	1.06	2.20	51.8	1.08	98.2	16.0	25.9
133 IV	1.18	0.76	1.94	60.8	1.55	1.15	1.05	2.20	52.3	1.10	98.2	15.2	24.5
136 XI	1.55	0.93	2.48	62.5	1.67	1.19	1.07	2.26	52.7	1.11	100.9	14.6	23.5
135 II	1.37	0.92	2.29	59.8	1.49	1.19	1.11	2.30	51.8	1.07	102.7	12.7	18.9
135 I	1.55	0.93	2.48	62.5	1.67	1.20	1.10	2.30	52.2	1.09	102.7	14.5	23.7
136 XI	1.62	1.00	2.62	61.8	1.62	1.32	1.17	2.49	53.0	1.13	111.2	13.7	21.8
106 III	1.62	1.01	2.63	61.6	1.60	1.33	1.22	2.55	52.2	1.09	113.8	14.9	23.5
106 I	1.60	0.98	2.58	62.0	1.63	1.40	1.19	2.59	54.1	1.18	115.6	12.7	20.8
106 III	1.62	1.00	2.62	61.8	1.62	1.43	1.18	2.61	54.8	1.21	116.5	11.3	18.3
131 I						1.50	1.30	2.80	53.6	1.15	125.0		
106 II	1.86	1.04	2.90	64.1	1.79	1.57	1.30	2.87	54.7	1.21	128.1	14.5	25.8

Table 20.

All contraction experiments on muscle fibres from *m. gluteus max.*

Column 12 states the height of compartment during contraction as a function of the mean value of compartment height at equilibrium length at rest ($2.24 \mu = 100$).

In columns 13 and 14 the shortening of A and the elongation of I during contraction is stated in per cent of the calculated length of A and I respectively during rest at the same degree of stretch. (Calculation conf. text p. 117).

Preparation	Rest					Contraction									Percentage of A Shortening of A	Percentage of I Elongation of I
	2	3	4	5	6	A (in μ)	I (in μ)	A (in μ)	I (in μ)	$(A + I)$ (in μ)	$\frac{A}{A + I} \times 100$	$\frac{I}{A + I}$	Degree of Stretch Length (Equilibrium at Rest = 100)	12	13	14
51 I	1.28	0.81	2.09	61.2	1.58	1.10	0.97	2.07	53.2	1.13	92.8	13.1	20.6			
51 II						1.22	1.05	2.27	53.8	1.16	101.8					
51 II	1.73	0.97	2.70	64.1	1.78	1.35	1.13	2.48	54.4	1.19	111.2	14.5	25.3			
52 IV	1.57	0.94	2.51	62.5	1.67	1.37	1.11	2.48	55.2	1.23	111.2	11.7	19.5			
52 IV	1.58	0.90	2.48	63.7	1.75	1.36	1.16	2.52	54.0	1.17	113.0	15.2	26.7			
50 V	1.57	0.99	2.56	61.3	1.59	1.37	1.18	2.55	53.7	1.16	114.3	12.4	19.7			
53 III	1.83	1.04	2.87	63.7	1.76	1.42	1.14	2.56	55.4	1.25	114.8	12.1	20.5			
52 V	1.84	1.03	2.87	64.1	1.79	1.42	1.24	2.66	53.4	1.15	119.3	16.0	28.0			
53 II	2.02	1.07	3.09	65.4	1.89	1.44	1.23	2.67	53.9	1.17	119.7	16.3	29.5			
53 II	2.06	1.13	3.19	64.6	1.83											
52 V						1.46	1.22	2.68	54.5	1.20	120.2					
48 II	1.65	1.01	2.66	62.0	1.63	1.43	1.27	2.70	53.0	1.13	121.1	14.4	23.4			
52 III	1.70	0.92	2.62	64.9	1.85	1.51	1.22	2.73	55.3	1.24	122.4	15.2	28.4			
49 IV	1.74	1.04	2.78	62.6	1.67	1.48	1.26	2.74	54.0	1.17	122.9	13.6	22.7			
51 II	1.83	1.01	2.84	64.4	1.81	1.45	1.29	2.74	53.0	1.12	122.9	16.1	27.7			
50 V	1.58	0.95	2.53	62.4	1.66	1.54	1.24	2.78	55.4	1.24	124.7	13.6	23.2			

52 II	1.76	1.01	2.77	63.5	1.74	1.51	1.30	2.81	53.7	1.16	126.0	15.5	27.1
53 III	1.81	1.04	2.85	63.5	1.74	1.56	1.28	2.84	54.9	1.22	127.4	13.5	23.6
50 IV	1.98	1.04	3.02	65.6	1.90	1.52	1.34	2.86	53.0	1.13	128.2	18.8	35.4
50 IV	2.03	1.08	3.11	65.3	1.88								
48 I	1.81	1.06	2.87	63.1	1.71	1.62	1.28	2.90	55.8	1.26	130.0	11.7	20.1
50 VI						1.56	1.36	2.92	53.4	1.15	130.9		
50 VI						1.64	1.34	2.98	55.0	1.22	133.6		
52 I	1.60	0.91	2.51	63.7	1.76	1.70	1.35	3.05	55.8	1.26	136.8	14.1	26.1
50 II	2.07	1.14	3.21	64.5	1.82	1.76	1.35	3.11	56.6	1.30	139.4	11.8	21.2

Table 21.

All contraction experiments on muscle fibres from *m. serratus post.*

Column 12 states the height of compartment during contraction as a function of the mean value of compartment height at equilibrium length at rest ($2.23 \mu = 100$).

In columns 13 and 14 the shortening of A and the elongation of I during contraction is stated in per cent of the calculated length of A and I respectively during rest at the same degree of stretch. (Calculation conf. text p. 117).

before and after contraction, see figs. 23—26. Isolated, excised mammalian muscle fibres can only stand a few contractions, consequently it has not been possible systematically to obtain series of contraction experiments on the same fibre at various degrees of stretch, and it also proved impossible to obtain measurable microphotographs of contracted fibres at lengths exceeding 140. Figs. 23—26 show some of the short series which could be carried through.

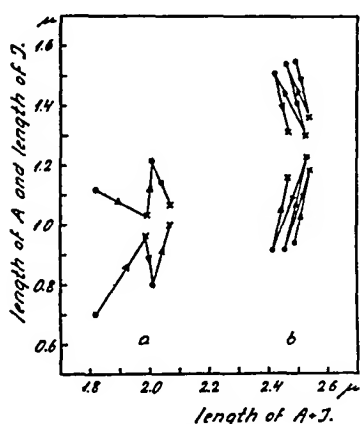


Fig. 23.

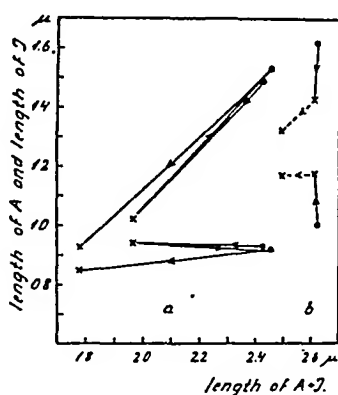


Fig. 24.

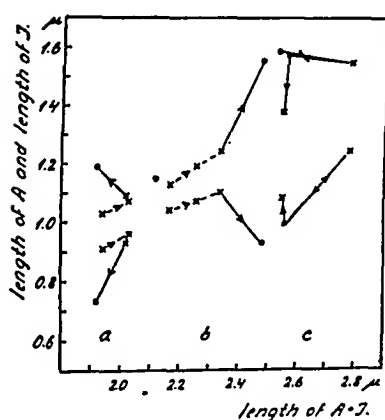


Fig. 25.

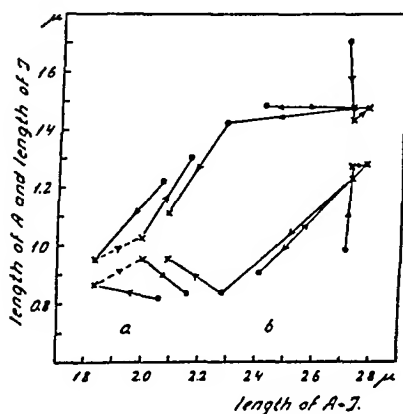


Fig. 26.

Figs. 23—26.

Absolute values of A and I during rest (•) and during contraction (×) as a function of the height of compartment. Experimental series with individual fibres. Fig. 23 a, b, fig. 24 a, b, fig. 25 a, b and fig. 26 a fibres from m. gluteus max. of guinea pig, fig. 25 c fibre from m. serratus post. of guinea pig and fig. 26 b fibre from m. serratus ant. of goat.

The arrows denote the sequence of the experiments.

Abscissa: length of A + I in μ .

Ordinate: length of A and I in μ .

As shown by tables 20—21 the contraction has not been absolutely isometric in all the experiments. This may be due to a varying degree of co-contraction on the part of the other fibres of the bundle when small bundles have been used, varying adhesion to the bottom of the chamber or the cover glass, etc. However, in the experiments no difference has been observed in the changes in the ratio $A:I$ during tetanic contraction whether the fibres were extended or shortened during contraction (conf. tables 20—21).

By means of the curves in fig. 17 the values of A and I at rest for the fibre in question at the measured contraction length can be calculated: calculated $A:I$ (at contraction length) =

$$\frac{\text{measured } A:I \times \text{mean value of } A:I \text{ (at measured contraction length)}}{\text{mean value of } A:I \text{ (at measured resting length)}}$$

The two last columns of tables 20 and 21 state the shortening of A and the elongation of I in per cent of calculated or measured values of A and I at rest at the same degree of stretch of the fibre concerned.

The mean values of the contraction experiments within the different degrees of stretch are given in tables 17 and 18.

The change of the ratio $A:I$ during contraction is somewhat different in the different fibres. The material as a whole shows that the percentage change in A and I during isometric contraction is the same for fibres from *m. gluteus max.* and *m. serratus post.* and fairly independent of the degree of stretch, conf. tables 17 and 18 and fig. 27. On an average A is shortened 14 per cent, while the elongation of I is 21 per cent during contraction when calculated on the basis of all the contraction experiments on fibres from *m. gluteus max.* The corresponding values for *m. serratus post.* are 15 per cent and 25 per cent.

When recording the mechanical properties of mammalian muscle fibres a "contraction equilibrium length" of 72μ — corresponding to a height of compartment of 1.60μ — is found by release of contracted fibres to tension zero. To correlate this observation with the histological investigations a number of experiments have been performed on compressed fibres from *m. gluteus max.* With fibres at rest a height of compartment as low as 1.82μ may be reached. During contraction of "compressed" fibres a height of compartment as low as

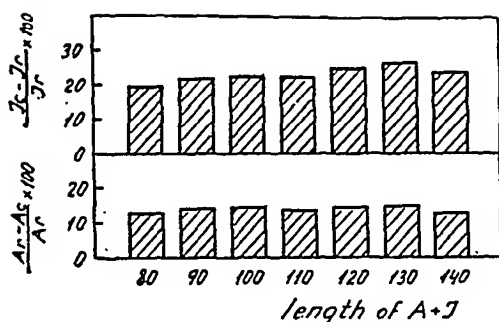


Fig. 27.

Percentage shortening of A and elongation of I during contraction at different degrees of stretch.

Mean values from all contraction experiments on muscle fibres from m. gluteus max. and m. serratus post.

Abscissa: height of compartment (equilibrium length = 100).

Ordinate: elongation of I in per cent of I's resting value at the same length of fibre.

shortening of A in per cent of A's resting value at the same length of fibre.

1.70 μ has been recorded (table 20). Even in these experiments, which are discussed in details in chapter V. D, the I-substance is elongated during contraction in proportion to its value at rest. The decrease of the equilibrium length of the muscle fibres which occur during contraction is thus confined to the A-substance, and the A-substance always constitutes a larger part of the compartment than the I-substance.

The mean results of all the experiments are stated in figs. 28 and 29 which represent the ratio A:I in the individual muscle compartments at rest and during contraction at different degrees of stretch.

Changes in the Ratio A:I during Single Contraction as a Function of Time.

The above mentioned contraction experiments have dealt with tetanic contractions of short duration. The morphological changes in mammalian muscle fibres during single contraction have also been examined and, by using *Buchthal & Knappeis'* set up (1943), it has been possible to record the changes in relation to the time by means of a photographic exposure for every 10 msec. The duration of a single contraction of mammalian muscle fibre is, however, only 30—40 msec.

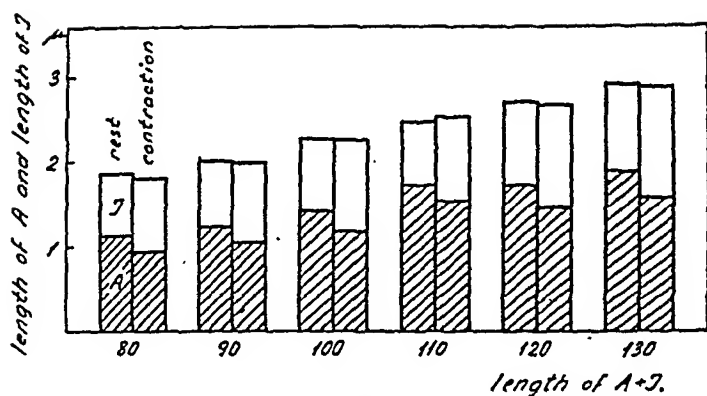


Fig. 28.



Fig. 29.

Figs. 28—29.

Absolute values of height of compartment, A and I during rest and during contraction at different degrees of stretch. Mean values of all experiments on fibres from *m. gluteus max.* (fig. 28) and *m. serratus post.* (fig. 29).

Abscissa: Height of compartment (equilibrium length = 100).

Ordinate: Height of A and I and A + I in μ .

so that the maximum number of recordings to be obtained during a contraction is three to four — in spite of the high frequency of the exposures. The course of the curve between measuring points is therefore more or less constructed, but the experimental results are so uniform that they yield a relatively accurate picture of the actual conditions.

The value of the ratio A : I at rest immediately before contraction is determined by four different measurements. The course of the actual contraction phase is the least well defined part of the curve, being determined by only one value. The procedure chosen has been to consider the time half-way between the last resting value and the first contraction value as

the beginning of the contraction, as this will probably give minimum error. The “active” part of the contraction is determined by a straight line through this point and the first measured contraction value. The relaxation phase of the contraction is usually recorded by means of two exposures, that is by two values, consequently the gradient of this phase is better defined, the same applies to the subsequent state of rest which is measured on four to five different photographs.

Figs. 30, 31 and 32 show three of these individual experiments. Fig. 33 shows a mean curve of three different single

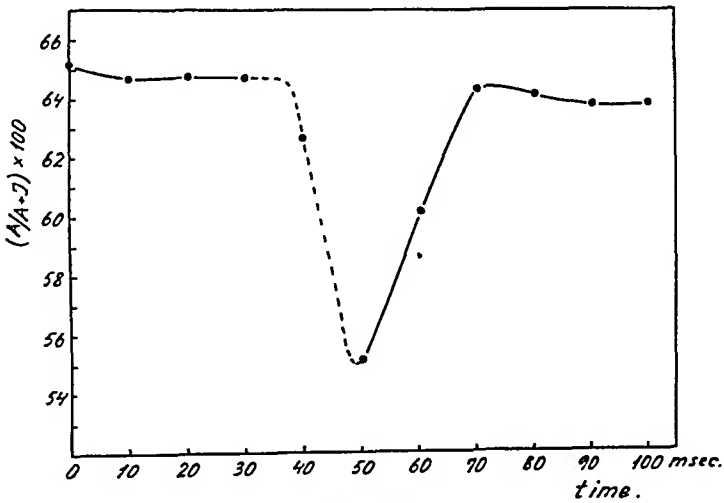


Fig. 30.

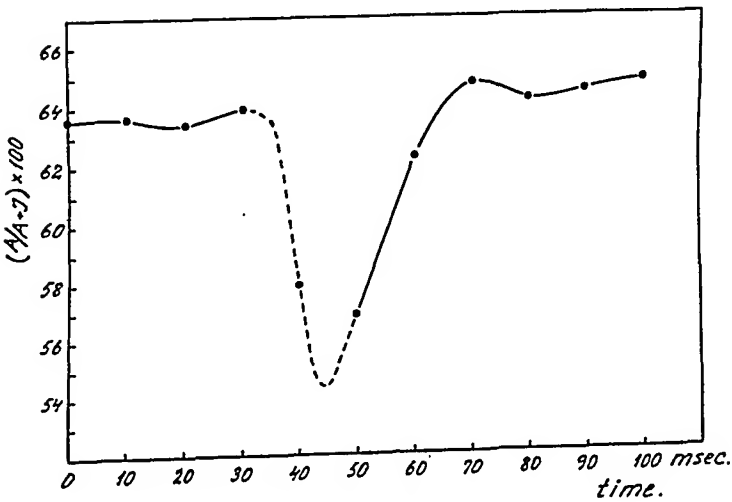


Fig. 31

contractions of the same fibre at the same length. The peak of the contraction has been taken as a common zero point as regards time when calculating the mean curve. The active phase of the single contraction seems to be of a shorter duration than the relaxation phase — which might be expected, and which is also confirmed by investigations of the mechanical properties of the fibres. The relative shortening of A is fairly equally pronounced during single contraction and tetanic contraction. On an average A is shortened by 14 per cent during single contraction and by 14—15 per cent during tetanic contraction. The optical changes during single contraction has a duration of approximately 30 msec. The values of the ratio A:I at rest is fairly uniform before and after contraction, a small increase of the ratio A:I may occur immediately after the contraction. In chapter V A these diagrams will be compared to the variations of tension during single contraction.

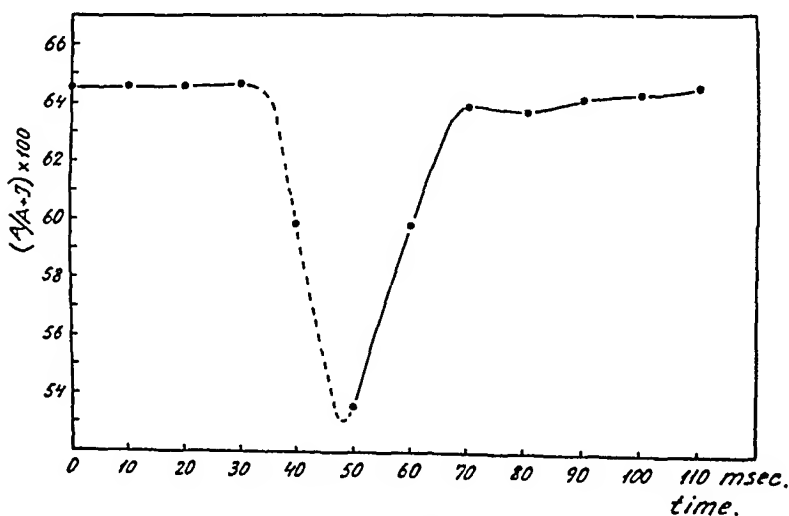


Fig. 32.

Figs. 30—32.

Variation in the ratio A : I during single contractions as a function of time. Three single experiments on muscle fibres from m. gluteus max. at length 115 (height of compartment 2.58 μ) in fig. 30, length 126 (height of compartment 2.70 μ) in fig. 31 and length 130 (height of compartment 2.91 μ) in fig. 32.

Abscissa: time in msec.

Ordinate: $(A/A + I) \times 100$.

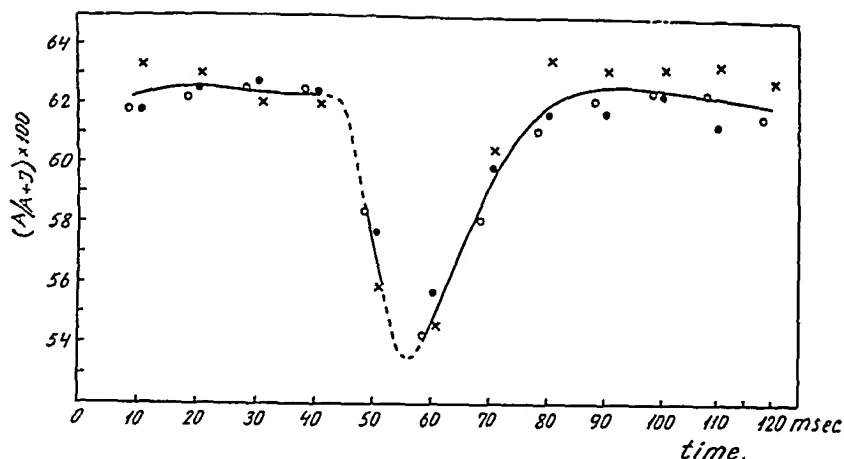


Fig. 33.

Variation in the ratio A:I during single contraction as a function of time. Mean curve of three single experiments on the same fibre from m. gluteus max. at the same degree of stretch; length 110 (height of compartment 2.64μ). See text p. 119.

Abscissa: time in msec.

Ordinate: $(A/A + I) \times 100$.

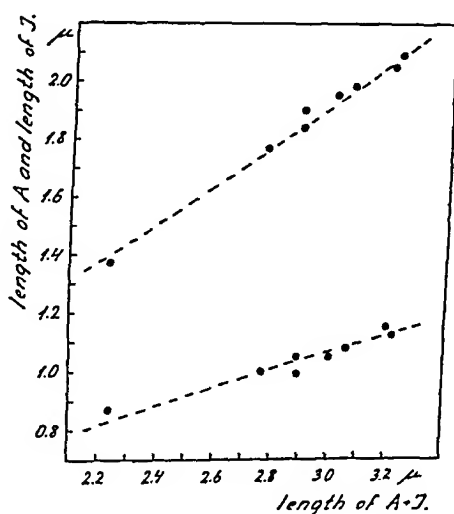


Fig. 34.

Muscle fibres from m. serratus post. of cat.

Length of A- and I-substance during rest as a function of the height of compartment.

The points plotted denote single experiments performed on five different muscle fibres. The dotted lines denote corresponding mean curves from experiments on fibres from m. gluteus max. of the guinea pig — for the purpose of comparison.

Abscissa: length of A + I in μ .

Ordinate: length of A (top curve) and I (bottom curve) in μ .

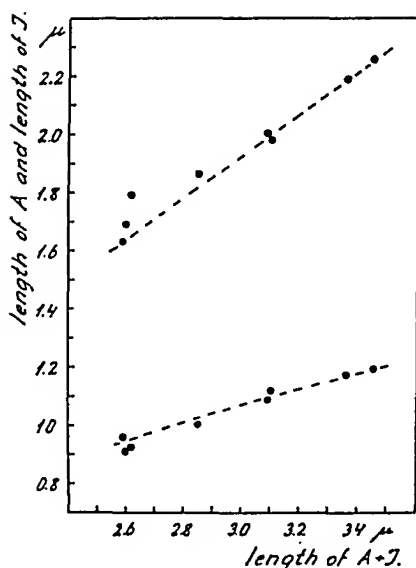


Fig. 35.

Muscle fibres from m. serratus post. of rabbit.

Length of A- and I-substance during rest as a function of the height of compartment.

The points plotted denote single experiments performed on three different muscle fibres. The dotted lines denote corresponding mean curves from experiments on fibres from m. gluteus max. of the guinea pig — for the purpose of comparison.

Abscissa: length of A + I in μ .

Ordinate: length of A (top curve) and I (bottom curve) in μ .

Investigation of Muscle Fibres from Various Mammals.

Solely for the purpose of orientation a number of experiments has been carried out on fibres from m. serratus post. of rabbit and cat (figs. 34 and 35). The equilibrium length has not been determined for these fibres, but the ratio A:I is exactly the same as that of the guinea pig fibres, and extension produces the same alterations in the structure. Finally muscle fibres from m. serratus ant. of goats suffering from congenital myotonia*) have been examined. Figs. 36—37 show that conditions in the fibres of these myotonic goats are the same as those in the muscles of guinea pigs, both as regards

*) These goats have also been used for various other physiological investigations, i. a. electro-myographic experiments (Buchthal & Clemmesen, 1941).

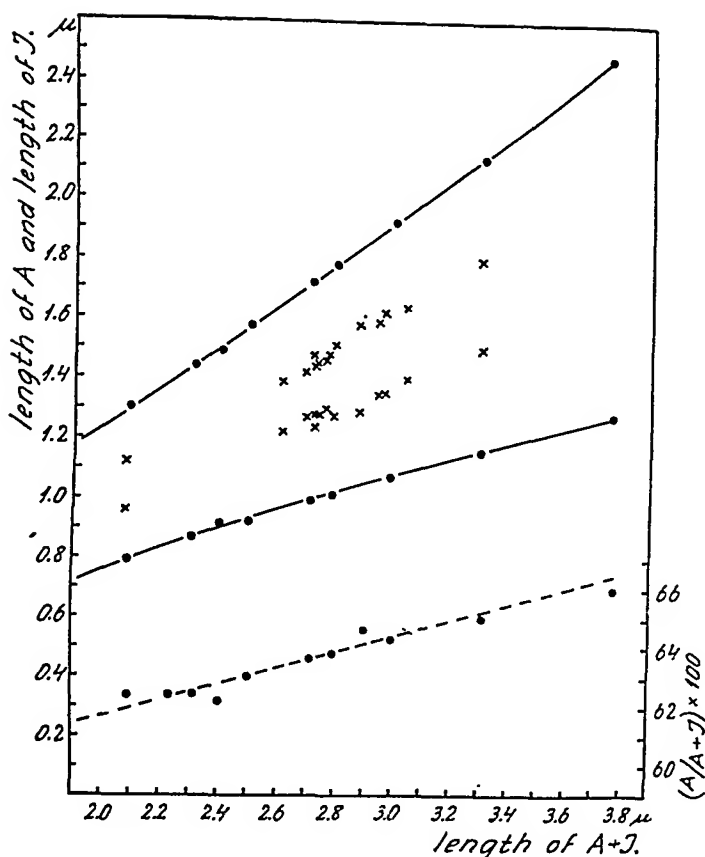


Fig. 36.

Muscle fibres from m. serratus ant. of goat.

Length of A- and I-substance during rest and during contraction as a function of the height of compartments. Each resting value (•) is a mean of four experiments. The contraction values (X) are results of single experiments.

Abscissa: length of A + I in μ .

Ordinate: (to the left) length of A (top curve) and I (bottom curve) in μ .
(to the right) $(A/A + I) \times 100$.

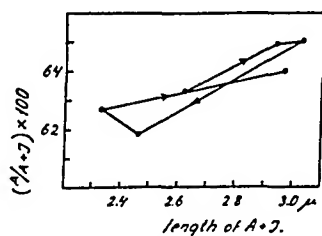


Fig. 37.

The ratio A : I as a function of the degree of stretch of the muscle fibres. Experimental series with the same resting muscle fibre from m. serratus ant. of goat.

The arrows denote the sequence of the experiments.

Abscissa: length of A + I in μ .

Ordinate: $(A/A + I) \times 100$.

height of compartment, ratio A:I and changes in the ratio A:I during stretch and during contraction. Hence it may be assumed that there is consistency also between muscles from normal goats and from guinea pigs.

Discussion.

The experiments have shown that the height of compartment at equilibrium length is practically uniform in the various muscles of the guinea pig. As further the ratio A:I is fairly uniform in muscle fibres from guinea pig, rabbit and goat at the same height of compartment, it may be assumed that the height of compartment at equilibrium length is likewise of the same magnitude in the various mammals. *Krause* (1868), *Hensen* (1868) and *Engelmann* (1873) mention that this uniformity in height of compartment even applies to all vertebrates, and in muscles from frogs (*m. semitendinosus*) and lizards *Buchthal*, *Knappeis* & *Lindhard* (1936) have found the height of compartment at equilibrium length to be $2.18 \pm 0.1 \mu$ and 2.24μ respectively — i. e. almost the same figure as in mammalian muscles. Very few of the previous investigators have defined the degree of stretch of the muscle accurately and for this reason the statements vary considerably. As examples the following may be mentioned: *Engelmann* (1880): abt. 4μ for stretched muscle fibres of vertebrates, *Stübel* (1920): 2.5μ for living frog fibres and *Frank* (1928): 2.4 — 2.5μ for living and 2.1 — 2.2μ for fixed frog muscle fibres. *Hürthle* (1930): 2.3μ for frozen and fixed frog muscle fibres, 2.7 — 3.0μ for living muscle fibres from the membrana basohyoidea of the frog, and *Speidel* (1939): 2.1μ for caudal muscles from the tadpole. *Sadow* (1936): $2.47 \pm 0.08 \mu$ for the *m. sartorius* of the frog at resting length, in situ, calculated on the basis of the diffraction spectra of the muscles.

Outside the vertebrates the height of compartment is more varying, as stated i. a. by *Engelmann* (1873) and *Speidel* (1938), and generally also larger: 10 and 17μ in insects according to *Engelmann* (1880), 5.8μ (4.8 — 6.6μ) in *Hydrophilus* according to *Hürthle* (1909), 6.3 and 6μ in *Astacus fluviatilis* (crayfish) and *Carcinus maenas* (crab) respectively, measured

on living muscle fibres at equilibrium length, according to *Knappeis, Lindhard & Topsøe-Jensen* (1940).

H. Brenner (1939) claims that the more rapid the muscle reacts the lower the height of compartment will be and the closer A/I will approximate one. When she finds that the height of compartment varies considerably within fibres from the same muscle, even within the same fibre, it must be due to defective and unfavourable experimental conditions, the focusing has varied, the fibres have exhibited local contractions, etc., an assumption which is confirmed by the highly varying $A:I$ ratio found by her. This appears e. g. from her tables 19 and 20 which show results of measurements on fibres from the same muscle (wing muscle of *Pieris*):

		$(A + I) \frac{A}{(A + I)} \times 100$
Average of 7 fibres,	4—5 μ thick	3.10 μ 54.5
— - 7 — ,	20—68 μ — , $A > I$	3.70 μ 54.5
— - 17 — ,	20—68 μ — , $A < I$	4.50 μ 42.7

The values are obtained by measuring ten compartments of each fibre, but her measurements from the same fibre show a coefficient of variation for the height of compartment of 28 and for the ratio $A:I$ — converted to the expression $(A/A + I) \times 100$ — of 16.6 (calculated according to her table I, fibre 6). Consequently the results of *H. Brenner* can hardly prove that the ratio $A:I$ differs in the different arthropod muscles, the dispersion of her experimental results being far too high.

As regards the ratio $A:I$ a difference is observed between *m. gluteus max.* and the other muscles of the guinea pig which have been examined, but the difference is so slight that it cannot be considered as real.

In the diagrams (figs. 15—16—17) the value of $(A/A + I) \times 100$ has been taken to increase linearly with the degree of stretch of the fibres. It is, however, possible that the shape of the true curve, which must of course be situated inside the hatched zone in figs. 18—19, will be slightly arched, a shape which expresses that the elongation of A is relatively smaller at low than at high elongations. Graphical curve fitting indicates that this holds for both materials — fibres from *m. gluteus max.* and from *m. serratus post.* — but the dispersion is too large to allow it to be shown with statistical accuracy

on the material as a whole. By the individual experiments it has also proved impossible to determine the percentage share of the height of compartment which falls on A with such an accuracy that the above mentioned slight variation in the value can manifest itself.

The muscles from the other mammals which have been examined show the same A:I ratio at rest as the muscles of the guinea pig, and probably the consistency does not only apply to mammalian muscles, but also to muscles from vertebrates. *Buchthal, Knappeis & Lindhard* (1936), who examined muscles from frogs and lizards with a technique similar to the one used for the present experiments on mammalian muscles, have reached the same result with regard to the ratio A:I. They found that the anisotropic substance always makes up the major part of the height of compartment in resting fibres and increases relatively more than the isotropic substance when being stretched i. e. has a smaller stiffness than I. In fibres from m. semitendinosus of the frog they measured A at equilibrium length to amount to 63 per cent of the height of compartment in their first material and to 61 per cent in a later material. (*Buchthal*, 1942, p. 49). The first material showed A = 65—67 per cent of the height of compartment at a length of the fibre of 140, in the later material the increase was hardly so pronounced, here = 63.5 per cent of the height of compartment at length 150. The cardiac muscles of the frog behave otherwise, here it is the I-substance which is most extensible, its stiffness is less than that of the A-substance, A = 60 per cent of the height of compartment at equilibrium length as against 58.8 per cent at length 170 (*Lundin*, 1944, p. 47).

Other investigators have found a slightly different A:I ratio for mammalian muscles, but this may i. a. have technical reasons. Most of the quantitative investigations have furthermore been based on single experiments and not on materials of any considerable size.

Engelmann (1873) states that the ratio A:I is fairly constant within the various animal classes and independent of the elongation of the fibres. *Frank* (1928) finds that A amounts to 46—50 per cent of the height of compartment in frozen and fixed muscles from frogs and that this value decreases when the fibres are stretched. *Hürthle* (1931, c) finds the same size, 45—50 per cent, while *Holz* (1932) states a

somewhat higher figure, 50—53 per cent; both investigators used living frog muscles fibres. *Jordan* (1934) finds that A is the substance which is most easily stretched (fixed preparations), while *Speidel* (1939) states that it is mostly I which is extended, an observation which he has made on living muscle fibres, quantitative results are, however, not given.

In living arthropod muscles it was found by *Knappeis, Lindhard & Topsøe-Jensen* (1940) that A amounts to 56 per cent of the height of compartment in fibres from *Astacus* as well as from *Carcinus*, and they also found that A is more extensible than I. According to *Engelmann* (1880) A amounts to 44 per cent of the height of compartment of living muscle fibres from insects, *Hürthle* (1909) on the other hand found that in *Hydrophilus* A amounts to 88 per cent of the height of compartment, and *Studnitz* (1935, a) measured A to be 57 per cent of the height of compartment. *Hürthle* found that the ratio A:I usually remains unaltered during stretch, in one third of the cases the increase of I was greater than that of A, but in these cases he assumed the fibres to be damaged.

When dealing with morphological changes during contraction the experimental results and the opinions become still more conflicting, even when only including investigations on living muscle fibres. An attempt will be made to elucidate the reason for this divergence.

First, however, the present investigations on mammals will be compared to the experiments performed by *Buchthal, Knappeis & Lindhard* (1936) on muscles of the frog. In muscles of the frog the actual shortening during contraction also takes place in the A-substance; A is shortened by 18 per cent, I elongated by 28 per cent, in case of tetanic, isometric contraction at equilibrium length; at length 116 the changes are 17 and 24 per cent respectively, and at length 141 they are 22 and 42 per cent. At equilibrium length A thus amounts to 52 per cent of the height of compartment during contraction. In later materials the changes in the ratio A:I during contraction found by *Buchthal & Knappeis* are slightly smaller (*Buchthal*, 1942, pp. 49—51), they state that the individual fibres exhibit individual quantitative differences, but it holds for all fibres that changes during contraction are smaller at moderate degrees of stretch (length 110—130) than at equilibrium length and at high degrees of stretch. They do not find any microscopically appreciable gradation of the changes in cross

striation during contraction corresponding to the gradation of tension exhibited by the muscle fibres during contraction at various degrees of stretch (*Buchthal & Knappeis*, 1943, b). Their experiments are consistent with the experiments on mammalian muscles in so far as it has also been impossible to recognize such a gradation here, but in mammals the percentage shortening of A is independent of the degree of stretch, while, as mentioned, the frog muscles exhibit a certain variation which is the same in the various experimental series (*Buchthal*, 1942, p. 50).

Holz' results from fibres of the frog (1932) are on line with these investigations, his results being that A is shortened during isotonic contraction. While in resting fibres A amounts to 50—55 per cent of the height of compartment it amounts to 43—47 per cent during contraction when the height of compartment has been reduced by 5—7 per cent, and 45—49 per cent when the height of compartment has been reduced by 20—25 per cent. *Sandow's* work on the diffraction spectra of muscles of the frog (1936) likewise shows that the changes during contraction must be opposed to the changes during extension, and that the changes may either consist in variations in the refractive indices of A and I or in variations in the A : I ratio.

In contrast to the above *Hürthle* (1931, c) observed that the ratio A : I is the same in resting and contracted fibres when investigating fibres of the frog fixed according to *Altman's* freezing technique; and *Feyel* (1936) likewise finds that A and I are both shortened, and that both are contractile — A more than I. His experiments are made on muscles of the frog.

In arthropod muscles *Engelmann* (1873, 1880) finds that the volume of A increases during contraction at the cost of I. *Hürthle* (1909) states that it is only A which is shortened, *Knappeis*, *Lindhard & Topsøe-Jensen* (1940) arrive at the same result, while *Studnitz* (1935, a) finds that I is shortened when A is shortened by more than 50 per cent. *W. J. Schmidt* (1937, p. 179) also adopts this theory (*Kölliker*, 1888) as he also observes I to be shortened at "maximal contractions".

Other authors are of the opinion that the contraction occurs exclusively in I, e. g. *Krause* (1873). *Frank* (1928) shows on fixed frog fibres that A is extended while I is shortened at isometric tetanic contraction. *Hürthle* (1930), who has checked these experiments, suspects the shortening of I here to be due

to artefacts. In tail muscles of the tadpole, examined in vivo, *Speidel* (1939) has observed narrowing both of A and I during "simple contractions", I narrowing relatively more than A and the shortening of the height of compartment amounting to 10 per cent. His so-called "simple contractions" are stated to last only $\frac{1}{10}$ sec. — consequently they are single contractions. He has, however, not measured the muscle fibres, for which reason his observations must be taken with reservation.

Engelmann (1873) has stated that the refractive indices of A and I are changed during contraction in such a way that A appears light in relation to I in contracted fibres. Several authors, e. g. *Merkel* (1873—1881), speak about so-called contraction bands (C) which arise when the anisotropic substance "divides" — corresponding to Q_H — and travels to Z where these contraction bands — of greater refractive index and higher stainability — are thus set up (conf. *Jordan*, 1920, 1933, 1934). *Bruno* (1930—1932) argues that this only happens in arthropods, in vertebrates the anisotropic substance concentrates about M during contraction. According to *d'Ancona* (1930) the contraction bands are produced by the I-granules being pressed against Z, while *W. J. Schmidt* (1937, p. 171) assumes the contraction bands to be due to intrafibrillar changes in Z as well as to compression of I-granules against Z, but especially the former.

In isolated living fibres of the frog (*Buchthal & Knappeis*, 1943) or in mammalian muscle fibres no reversal of the cross striation during contraction has been observed — it should be especially emphasized that no such observations have been made during the examinations of single contractions in which the time course of the changes in ratio A:I is recorded. Neither have *Sandow's* investigations on the diffraction spectra of muscle fibres (1936) furnished any evidence of a doubling of the cross-striation during the actual contraction phase which, according to *Merkel's* theory (1873), should happen.

Contraction bands are thus hardly a normal contraction phenomenon, but they are especially in evidence when the shortening of the fibres is not physiological — i. e. when shortening exceeds 45 per cent.

In chapter V it is mentioned that by releasing contracted mammalian muscle fibres to tension nil a shortening of 28 per cent is caused; in fibres from frogs *Buchthal* (1942, p. 24) found the corresponding figure to be about 30 per cent, while

the shortening in case of tensionless contraction is about 45 per cent.

Correspondingly *Speidel* (1939) finds that the images of "simple contractions" will occur when the shortening during contraction amounts to 25 per cent or less, while contraction bands are formed when the shortening exceeds 40 per cent. It is possible from the literature to quote several investigators who have made the same observations, *Stübel* (1920), f. inst. finds a "compound cross striation" with a height of compartment of $2.5\ \mu$ in living, resting fibres from frog muscles, this he considers to be normal, and a simple cross striation with a height of compartment of only $0.9\ \mu$, which he assumes to be an artefact — a kind of "supermaximal muscle contraction" which he only observes in "dying or dead muscle fibres". *Frank* (1928) mentions that shortening beyond 25 per cent during "contraction" gives quite different microscopical images, and *Ramsey & Street* (1940) find that irreversible changes both as regards cross striation and mechanical properties take place in the muscle fibres when they are induced to shorten by 60—70 per cent by means of electric stimulation.

As may be seen from the above the diverging conceptions of the histological picture of muscle contraction are not only caused by defective optical technique (errors in focusing, etc.), but also by artificial structural alterations being interpreted as normal contraction phenomena.

As no difference can be discerned in the height of compartment or the ratio A : I in fibres from m. obliquus abd. intern. whether excised or in situ — in the last case presumably with intact innervation — the experiments have yielded no confirmation of the theory that fibres in vivo possess a certain "tone" in the shape of a permanent state of contraction. *Speidel* (1938) on the other hand assumed that fibres in situ are in a "tonic" slightly contracted state, even if he could not histologically recognize any shortening of the sarcolemma.

The various hypotheses regarding the anatomical substratum of muscular tone is summarized in *Wohlfart's* paper (1937, pp. 14—22). Some investigators have f. inst. assumed the sarcoplasm to possess contractility (*Olivo*, 1925; *Häggquist*, 1931, pp. 164—165; *Speidel*, 1939) especially in the shape of tonic activity, a theory originally advanced by *Bottazzi* (1896) and which is still adopted by some investigators (i. a.

by *Riesser*, 1925, p. 232). By the present experiments on mammals nothing has been found to confirm such a contractility, and *Ryden & Wohlfart's* "proof" (1932) cannot be described as striking. They assume the sarcoplasm to be contractile on account of their findings that some muscles contain extremely few myofibrils, and in these muscles there would be excessive loss of power if the myofibrils were the only contractile elements. The theory already propounded by *Engelmann* (1873) that contractility is connected with birefringent substance should also be remembered, and, according to *Friedheim* (1931), muscle tissue cultivated in vitro does not exhibit contractility until cross striation and fibrillar structure can be discerned.

Conclusion.

The height of compartment and the ratio of anisotropic to isotropic substance in the individual compartment vary only slightly within the resting muscle fibre.

With regard to height of compartment as well as ratio A:I and changes in the latter during stretch and contraction conformity has been established between the muscles of the guinea pig examined, namely the m. gluteus max., m. serratus post. and m. obliquus abd. intern.

At equilibrium length the height of compartment amounts to 2.2 μ , this being a fairly reproducible value.

At equilibrium length the anisotropic substance amounts to 62—63 per cent of the height of compartment and the isotropic to 37—38 per cent.

During stretch of muscle fibres the length of the anisotropic substance increases more than that of the isotropic. When the fibres are stretched 50 per cent A amounts to 65—66 per cent of the height of compartment.

During isometric, tetanic contraction the shortening of the anisotropic substance is on an average 14—15 per cent, while the increase in height of the isotropic layer is 21—25 per cent.

The percentage change of the A- and I-substance during contraction is independent of the degree of stretch of the fibres.

During single contraction and tetanic contraction the changes are the same.

During single contraction the changes in the ratio A:I last, at most, 30—40 msec.

Muscle fibres from cat, rabbit and goat show the same microscopical images as those of the guinea pig.

The experiments on mammals have given the same results as the experiments by *Buchthal, Knappeis & Lindhard* performed with identical technique on muscle fibres from frogs and lizards. This indicates conformity between the muscles of the vertebrates.

The highly differing observations made by other authors may be explained by the use of fixed material, and, in case living muscle fibres have been used, by erroneous interpretation of the optical images and of artificial structural alterations are considered to be normal histological phenomena.

CHAPTER IV.

INVESTIGATIONS ON THE BIREFRINGENCE OF THE MUSCLE FIBRES.

Investigations of the minute structure of the muscular tissue have, as previously mentioned, preferably been carried out by means of polarization-optical, roentgen-spectroscopical or thermo-elastic methods. Among these methods the one directed upon the birefringence is technically the most convenient.

The experimental conditions of previous determinations of the birefringence have, however, been unfavourable, especially with regard to the quantitative determinations. When using whole muscles (*Hermann*, 1880; *v. Ebner*, 1882; *v. Muralt*, 1932; *Noll & Weber*, 1934 and *E. Fischer*, 1936, 1944) the heterogeneous structure of the muscles with the resultant varying and uncontrollable tension of the different muscle fibres, the varying contents of tendons, vessels, nerves and connective tissue will give rise to inaccurate results. And when fixed material is used (i. a. *Stübel*, 1923; *Noll & Weber*, 1934 and *E. Fischer*, 1936, 1944) the experimental results are compromised by the varying and often intense shrinking of the fibres produced by the fixatives. When isolated, living muscle fibres are examined, however, the experimental conditions become more reproducible. *Buchthal & Knappeis* (1938, 1944 and 1946) have carried out determinations of the birefringence in such preparations from cold-blooded vertebrates, frogs and lizards, and corresponding investigations on living mammalian muscle fibres will now be described.

The birefringence ($n_a - n_o$) is, as mentioned on p. 69, determined by the equation $(n_a - n_o) = \frac{\gamma\lambda}{d}$, where d is the

degrees of stretch for which reason the equation $x = d_2 \sqrt{f_2/f_1}$

can be used for converting the diameter d_2 , measured at length f_2 , to length f_1 , and the result may be compared with the diameter d_1 measured at this length. The ratio between the two measured diameters converted to the same length $\left(\frac{d_2 \sqrt{f_2/f_1}}{d_1} \times 100 \right)$ is thus an expression of the accuracy of

measurement and fibre deformation, i. e. deviation from the cylindrical shape; as previously shown the height of compartment can be measured fairly accurately. Fig. 38 shows the

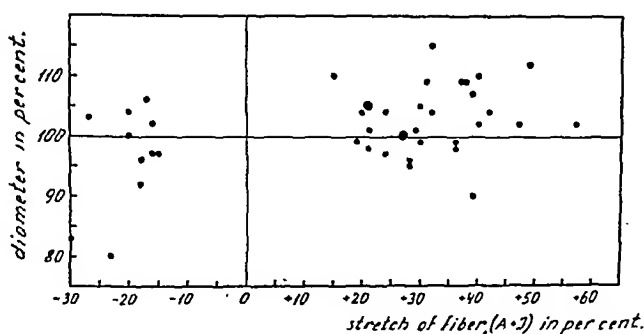


Fig. 38.

The ratio between the diameters of individual muscle fibres at two different degrees of stretch — as a function of stretch or relaxation respectively. The difference between the two diameters is corrected for the variation in diameter which a corresponding stretching of a cylinder would effect (see text p 135).

Preparations: 42 muscle fibres from m. gluteus max.

Abscissa: $\frac{f_2 - f_1}{f_1} \times 100$.

Ordinate: $\frac{d_2 \sqrt{f_2/f_1}}{d_1} \times 100$.

(f = height of compartment in μ , d = diameter in μ).

results of these diameter measurements. To avoid systematic errors some of the fibres have first been examined in relaxed condition and others first in stretched condition. There are slightly more values above the zero line in case of stretched fibres, slightly more below the zero line in case of relaxed fibres. This suggests that the muscle fibres have a tendency to flatten when being stretched.

To a certain extent it is possible to obtain a check on the cross section of the fibres by observing the shape of the deviation of the interference line in the Babinet's compensator which corresponds to the fibre cross section. A circular fibre will produce a regular arch-shaped deviation. In cases where the deviation has been irregular or asymmetrically arch-shaped the experiment has been discarded.

The fact that a correction factor is required for measurements of birefringence carried out with this technique will be discussed later on (see p. 143).

In table 22 the results of the determinations of birefringence on muscle fibres at equilibrium length (height of compartment $2.14\text{--}2.35\ \mu$) are summarized. 38 fibres from m. gluteus max. exhibit an average value of the birefringence of $(1.68 \pm 0.08) \times 10^{-3}$ ($p = 1$ per cent) at an average height of compartment of $2.28\ \mu$. The corresponding value for fibres from m. serratus post. (17 fibres, average height of compartment $2.27\ \mu$) amounts to $(1.64 \pm 0.12) \times 10^{-3}$. The difference between these two mean values is within the standard error of the mean. If we consider the two experimental series as a whole (55 fibres, average height of compartment $2.28\ \mu$) the mean figure for the birefringence will be $(1.67 \pm 0.06) \times 10^{-3}$.

In order to ascertain whether there is any difference between the birefringence of thin and thick muscle fibres the ratio: phase difference ($\gamma\lambda$) to thickness of fibre (d) has been plotted in figs. 39 and 40 for muscle fibres at equilibrium length and for the whole material irrespective of degree of stretch. It appears from these diagrams that a relatively low birefringence has been found for thick fibres and a relatively high one for thin fibres. It should be emphasized that this phenomenon does not only apply to the material in toto, but also to the fibres at equilibrium length. Consequently the difference is not caused by extension — what might be expected, as the material in toto, as a matter of course, contains a larger percentage of stretched muscle fibres among the thin fibres than among the thick ones — but indicates a certain flattening of the thick fibres.

The relation of the birefringence to the degree of stretch of the muscle fibres can be illustrated by means of experiments on the individual fibres and by investigation of the material as a whole. Fig. 41 shows some examples of the alteration of fibre diameter, phase difference and birefringence

Number	Height of Compartment (in μ)	Thickness of Fibre (in μ)	Phase Difference ($\gamma\lambda$) (in $m\mu$)	Birefringence ($n_a - n_o$) $\times 10^{-3}$		
	Mean Value	Mean Value	Mean Value	Mean Value	Disper- sion	Standard Error of the Mean ($P = 1\%$)
<i>Equilibrium length</i> (2.14—2.35 μ)						
m. gluteus max.	38	2.28	53.9	90.0	1.68	0.08
m. serratus post.	17	2.27	60.8	99.2	1.64	0.12
m. gluteus max. + m. serratus post.	55	2.28	56.0	92.9	1.67	0.06
<i>Elongation beyond 5 per cent</i>						
m. gluteus max.	124	2.78			1.73	0.05
m. serratus post.	19	2.75			1.71	0.07
m. gluteus max. + m. serratus post.	143	2.78			1.73	0.04

Table 22.

Birefringence of muscle fibres from m. gluteus max. and m. serratus post. at equilibrium length and during stretch.

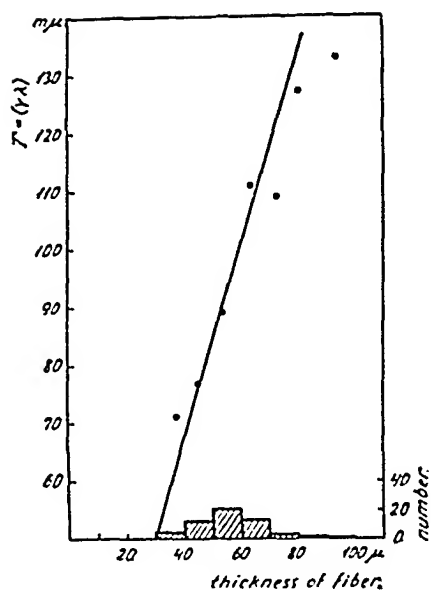


Fig. 39.

Phase difference as a function of thickness of fibre at equilibrium length of the muscle fibres.

Preparation: 55 muscles fibres from m. gluteus max. and m. serratus post. The height of the hatched columns denotes the number of experiments by means of which the individual points have been determined.

The line represents the calculated phase difference at different thickness of fibre with a birefringence of 1.67×10^{-3} .

Abscissa: diameter of fibre in μ .

Ordinate: (to the left) phase difference ($\gamma\lambda$) in $m\mu$.

(to the right) number of experiments.

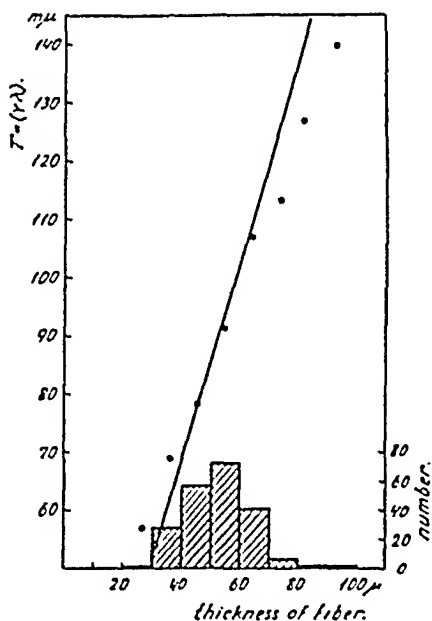


Fig. 40.

Phase difference as a function of thickness of fibre, measured on muscle fibres independent of their degree of stretch.

Preparation: 117 muscle fibres from m. gluteus max. and m. serratus post. The line represents the calculated phase difference at different thickness of fibre with a birefringence of 1.72×10^{-3} .

Abscissa: diameter of fibre in μ .

Ordinate: (to the left) phase difference ($\gamma\lambda$) in $m\mu$.

(to the right) number of experiments.

during stretch of the same fibre. As far as possible the same part of the fibre has been used for the various measurements, this being secured either by following the actual extension under the microscope or by means of some distinguishing marks on the fibre — e. g. fibrils of connective tissue or the like. When the fibre is stretched the phase difference decreases, and at the same time the fibre diameter is diminished, while the birefringence — being a function of these two values — may behave in various ways, as seen from the examples

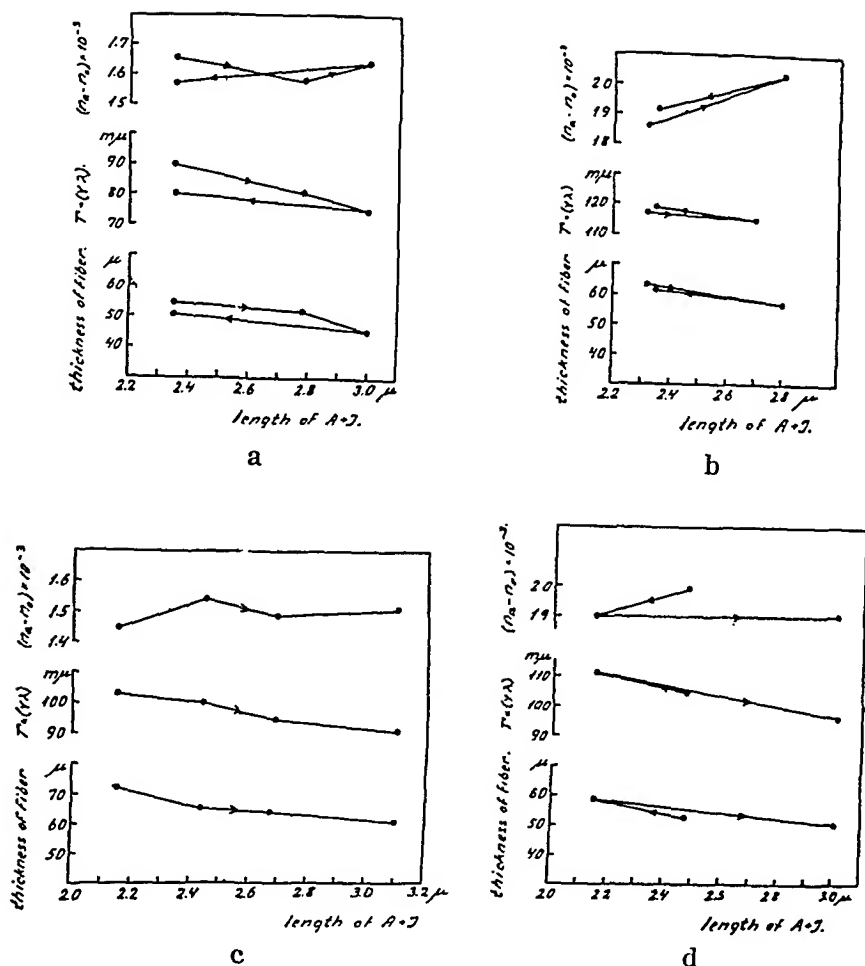


Fig. 41.

Diameter of fibre, phase difference and birefringence as a function of stretch. Four individual experiments.

Preparations: muscle fibres from m. gluteus max. (a, b, c) and from m. serratus post. (d).

The arrows indicate the sequence of the measurements.

Abscissa: height of compartment in μ .

Ordinate: diameter of fibre ($= d$) in μ .

phase difference ($\gamma\lambda$) in $m\mu$.

birefringence $(n_a - n_0) \times 10^{-3}$.

shown (fig. 41). Most frequently the birefringence remains unaltered, but there are cases in which it increases as well as cases in which it decreases when the fibres are stretched; the variation of the birefringence in the individual experiments thus merely seems to be an expression of the dispersion of the technique used. In fig. 42 the mean values for the whole

material are shown. The latter also exhibits a fairly unaltered birefringence at the various degrees of stretch. Only when the fibres are stretched beyond length 150 does an increase of the birefringence occur, but the experiments are too few to justify this being established as a fact, neither is the difference statistically valid.

In muscle fibres below equilibrium length a relatively high birefringence has been found, namely 1.83×10^{-3} , this being

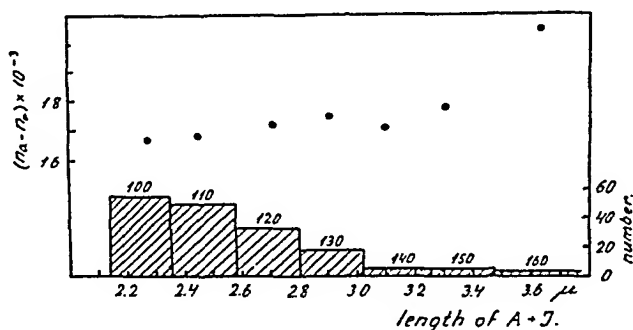


Fig. 42.

Birefringence as a function of degree of stretch.

Preparation: 117 muscle fibres from m. gluteus max. and m. serratus post.

The figures above the hatched columns denote length of fibre (equilibrium length = 100).

The height of the hatched columns represents the number of experiments within each group (length 95—105, 105—115 etc.).

Abscissa: height of compartment in μ .

Ordinate: (to the left) birefringence $(n_a - n_o) \times 10^{-3}$.

(to the right) number of experiments.

the mean of nine fibres at an average fibre length of 93. The high value measured does hardly represent an actual increase of the birefringence, but is no doubt due to a deformation — wrinkling or the like — of the muscle fibres caused by the compression, which results in an erroneous measurement of d .

The birefringence of the whole material, independent of the degree of stretch, amounts to $(1.72 \pm 0.04) \times 10^{-3}$.

When the muscle fibres were injured *Buchthal & Knappeis* often found a temporary increase of the birefringence — values up to 2.3×10^{-3} . Such high figures have not been found by experiments on mammalian muscle fibres — the latter always exhibited a decrease of the birefringence when injured.

Discussion.

Buchthal & Knappeis (1938) found proportionality between phase difference ($\gamma\lambda$) and thickness of fibre (d), their diagrams only showed a somewhat high value of the birefringence of thick fibres unlike the observations of mammalian muscle fibres which show relatively high anisotropy of the thin fibres and relatively low anisotropy of the thick fibres. This fact may be caused by the thick fibres being flattened more than the thin ones in mammalian muscle fibres. The measured value of d is consequently higher than the actual one and the birefringence of the thick fibres have thus been found too low. The possibility that the thick muscle fibres have a relatively smaller content of myofibrils than the thin ones cannot be completely excluded either. It is asserted, e. g. by *Schieffer-decker & Schultze* (1903) that in case of activity-hypertrophy of the muscle fibres it is preferably the sarcoplasm which is increased, only to a minor degree the myofibrils. According to *E. Fischer* (1940) there is no definite relationship between decrease of fibre diameter and decrease of phase difference in case of muscle atrophy.

Hermann (1880) and *v. Ebner* (1882, p. 138) found different anisotropy in different muscles of the same animal. The present investigation shows no such variation, at any rate the difference between the results obtained by determining the birefringence of muscle fibres from two different muscles is smaller than the dispersion. The fact that the ratio $A:I$ is practically identical in the two different muscles is quite in agreement with the uniform birefringence of the two muscles, but identical $A:I$ ratio need not, of course, result in identical birefringence.

As regards the magnitude of the birefringence the results of the present investigation show good conformity with those of *Buchthal & Knappeis* (1938) which are obtained with the same experimental technique. *Buchthal & Knappeis* found the average value of the birefringence of living isolated fibres from *m. semitendinosus* of the frog to be $(1.70 \pm 0.016) \times 10^{-3}$. In the guinea pig the average figure for the birefringence of isolated fibres was $(1.67 \pm 0.06) \times 10^{-3}$. The difference is so small that it is not necessarily real, being within the standard error of the mean. The same reasoning as mentioned above may also be applied here: the fact that the birefringence of

muscle fibres of frogs and mammals measured by the same method has been found to be the same confirms the correctness of the histologically observed conformity between the A : I ratio of the cross striated muscles of the two different animal classes — and vice versa.

In later investigations (not yet published), which have been carried out after my birefringence measurements have been concluded, *Buchthal & Knappeis* use the improved technique, already mentioned, of direct measurement of the thickness of the birefringent layer, they now find a higher value of the birefringence of the muscle fibres of frogs than in their first experiments, namely a birefringence of $(2.01 \pm 0.048) \times 10^{-3}$ as against the previous result $(1.70 \pm 0.016) \times 10^{-3}$ — i. e. a figure which is 18.5 per cent higher. This difference suggests that by the experimental technique used in the first investigations a slight deformation of the muscle fibres has been produced. The relatively low birefringence of the thick fibres observed during the present investigation may, as discussed above, be an indication in the same direction, and the check measurements of the diameter likewise suggest a tendency to deformation of the fibre cross section dependent on the longitudinal tension of the muscle fibres.

Buchthal & Knappeis (1946) have carried out a closer investigation of these conditions and demonstrated that the fibres become slightly deformed by resting against the slide. The magnitude of this deformation is such as to cause an error in the determination of the birefringence of 17—18 per cent. It has already been mentioned in chapter I that *Buchthal, Deutsch & Knappeis* (1946) also observed that the cross section of muscle fibres from frogs is far from circular, but such irregularity in the cross section of the fibres will not influence the mean value of the birefringence determinations. As a consequence of the preparation technique the fibres are equally often orientated with the largest as with the smallest diameter at right angles to the muscle chamber, so that the errors of the individual determinations will be equalized when the material is sufficiently large.

As a consequence of these investigations by *Buchthal & Knappeis* a correction should be introduced in the birefringence measurements obtained by means of the technique used by me. The mammalian muscle fibres show a birefrin-

gence of 1.98×10^{-3} when a correction based on the frog fibre-experiments is introduced.

The results obtained by other previous quantitative determinations of the birefringence of muscle tissue vary between 1.70 and 3.00×10^{-3} . Among such investigations the following may be mentioned:

v. Ebner (1882, p. 138): living m. sartorius of frog.

Birefringence: 2.07×10^{-3}

Stübel (1923): muscles of the frog, fixed by means of alcohol and 10 per cent formol respectively.

Birefringence: 3.00×10^{-3} and 2.20×10^{-3}

Noll & Weber (1934): fixed fibres from various muscles of the frog and living m. cutaneous pectoris in toto also of the frog.

Birefringence: 2.30×10^{-3}

T. Y. Liang (1936): living, isolated fibres from m. sartorius of the frog.

Birefringence: 1.90×10^{-3}

Buchthal & Knappeis (1938): living, isolated fibres from m. semitendinosus of the frog,

Birefringence: $(1.70 \pm 0.016) \times 10^{-3}$

living, isolated muscle fibres of the lizard.

Birefringence: 1.81×10^{-3}

Buchthal & Knappeis (1946): living, isolated fibres from m. semitendinosus of the frog.

Birefringence: $(2.01 \pm 0.048) \times 10^{-3}$

E. Fischer (1944): the following surviving whole muscles:

m. sternohyoideus of the dog,

Birefringence: $(2.48 \pm 0.07) \times 10^{-3}$

m. sartorius of the dog,

Birefringence: $(2.60 \pm 0.11) \times 10^{-3}$

m. rectus abdominis of the dog,

Birefringence: $(2.57 \pm 0.15) \times 10^{-3}$

cross striated muscle tissue from oesophagus of the dog,

Birefringence: $(2.54 \pm 0.09) \times 10^{-3}$

m. rectus abdominis of the mouse,

Birefringence: $(2.61 \pm 0.02) \times 10^{-3}$

and various fixed mammalian muscles,

Birefringence: $(2.25 - 2.58) \times 10^{-3}$

a few check experiments on muscles of the frog,

Birefringence: 2.24×10^{-3}

In chapter I the polarization-optical conditions of the individual microscopical, structural elements of the muscle fibres are dealt with. It may be emphasized that mammalian muscle fibres, just as muscle fibres from frogs, impart a finely serrated contour to the arch-shaped deviation of the interference line in the Babinet's compensator, and this must mean that the birefringence is caused by the longitudinal structural elements of the muscle fibre, i. e. the myofibrils.

According to the present material the birefringence is independent of the stretch of the fibres — at any rate as long as the degree of stretch does not exceed 60 per cent of the equilibrium length of the fibres. *Hermann* (1880) could not observe any changes in the constants of the muscles during stretch. He certainly found that the phase difference decreased more than the calculated thickness of the fibre bundle, but this he explained by assuming the fibre bundle to be flattened when stretched, as he found fibre bundles of the same thickness, but at different degrees of stretch, to have the same birefringence. *v. Ebner* (1882, pp. 80—88) found different alterations of the birefringence when stretching different muscles, the slightly anisotropic muscles exhibited increase of the birefringence, the more birefringent muscles were unaltered by stretch. *Buchthal & Knappeis'* first investigations (1938) did not show any increase of the birefringence on extension either, the birefringence being 1.75×10^{-3} for stretched fibres, as against 1.70×10^{-3} for fibres at equilibrium length. They did not examine fibres elongated more than 45 per cent. The later material (1946) on the other hand showed a small increase of the birefringence during stretch, 3 per cent for each 25 per cent of stretch, but they also recorded considerable variations within the individual experiments. *E. Fischer* (1944) observed a slight increase of the birefringence when whole, living muscles were stretched — the material used being mostly the m. rectus abdominis of the mouse. At "normal" length the birefringence of this muscle was $(2.61 \pm 0.02) \times 10^{-3}$, at slight elongation $(2.68 \pm 0.02) \times 10^{-3}$, and at 27 per cent of stretch $(2.79 \pm 0.03) \times 10^{-3}$. The difference is statistically valid and amounts to an increase of the birefringence of 4 per cent at 25 per cent of stretch of the fibres. During stretch of smooth muscles a pronounced increase of the birefringence will, according to *E. Fischer* (1936, 1944), likewise occur. My material also suggests such an increase, but, as already mentioned, the

material alone is not sufficient to prove the existence of this increase — it is only at the higher degrees of stretch that the increase in the birefringence becomes more obvious.

Whether it is the rod birefringence or the crystalline birefringence of the myofibrils which is increased when the fibre is stretched cannot be disclosed by examination of living muscle fibres — for this purpose imbibition experiments are required. The quoted investigations by *Buchthal & Knappeis* (1940) on the diffraction spectra of muscle fibres, which shows that the orientation of the micellae is not strictly parallel at equilibrium length and does not become parallel until the fibres are stretched 10—30 per cent, is not inconsistent with the birefringence investigations. A slight deviation from the parallel orientation of the micellae will not influence the birefringence perceptibly.

On myosin threads, which are not, however, directly comparable to muscle fibres, *H. H. Weber* (1934, b) found that the initial increase in the birefringence during stretch may be considerable and is due to increase of the rod birefringence, the orientation of the micellae becoming parallel; the later, less pronounced, increase is caused by an increase of the crystalline birefringence. In smooth muscle fibres *E. Fischer* (1938) found that the birefringence increases when the fibres are stretched, and this increase was supposed essentially to be due to the crystalline birefringence, as the rod birefringence is at its optimum already at “normal” length of the fibres. By the imbibition method *E. Fischer* examined cross striated mammalian muscles fixed at different lengths, and he found that while the rod birefringence and crystalline birefringence are equally much influenced by alterations of the length of such muscle fibres, the smooth mammalian muscle fibres show much greater decrease of the rod birefringence than of the crystalline birefringence when shortened. As, however, the preparations have been fixed while still contractile these experimental results cannot be assumed to express any “physiological” function of the muscle fibres — conf. the fixation artefacts mentioned elsewhere.

It has not been possible, with the available methods, to investigate the changes in the birefringence during contraction. Such investigations have, however, been performed on living frog muscles e. g. by *v. Muralt* (1932) and on living,

isolated frog fibres by *Buchthal & Knappeis* (1938) and *Buchthal, Deutsch & Knappeis* (1944, 1946), as mentioned in chapter I.

Conclusion.

On isolated, living, cross striated muscle fibres of the guinea pig the birefringence has been measured to be $(1.67 \pm 0.06) \times 10^{-3}$ at equilibrium length.

Later investigations by *Buchthal, Deutsch & Knappeis* (1946) with a more accurate technique have shown that the birefringence, as measured by the technique employed by *Buchthal & Knappeis* (1938) and by the author of the present work, is 18.5 per cent too low. The exact birefringence of muscle fibres of the frog was measured by *Buchthal & Knappeis* (1946) to be 2.01×10^{-3} . The corrected birefringence of mammalian muscle fibres is 1.98×10^{-3} .

The birefringence is measured to be slightly lower for thick muscle fibres than for thin ones, a fact which is assumed to be caused by a slight flattening of the thick fibres or, possibly, a relatively smaller content of birefringent substance in the latter.

Fibres from various muscles — m. gluteus max. and m. serratus post. — show practically the same birefringence: $(1.68 \pm 0.08) \times 10^{-3}$ and $(1.64 \pm 0.12) \times 10^{-3}$ respectively.

The birefringence is caused by the longitudinal structural elements of the muscle fibres — the myofibrils.

The birefringence of muscle fibres of the guinea pig is of the same magnitude as the birefringence of muscle fibres of the frog — the latter being $(1.70 \pm 0.016) \times 10^{-3}$ (according to *Buchthal & Knappeis*, 1938) when measured with the same technique.

This confirms the fact that histologically the ratio A:I is found to be the same in muscles from these two animal classes.

The present material does not prove that the birefringence increases when the muscle fibres are stretched, but it indicates such an increase.

CHAPTER V.

INVESTIGATIONS ON THE MECHANICAL PROPERTIES OF THE MUSCLE FIBRES.

The mechanical properties of the muscle fibres can be examined by means of static, dynamic or semi-dynamic experiments. Through static experiments the relationship between length and tension of the fibres during rest and contraction is examined after complete stabilization, i. e. when the elastic after-effects have ceased. In case of dynamic experiments the recording of length and tension takes place during the actual movements, and when the latter occur at a reduced speed — the extension lasting for 1—10 sec. — the experiments are called semi-dynamic.

When examining the mechanical properties of muscle fibres, distinction should be made between elasticity, viscosity and plasticity of the fibres.

A body is referred to as elastic if it alters its shape, when external forces are applied to it, and regains its original shape, when the forces cease to act. The elasticity of a substance is usually expressed by its modulus of elasticity

$$\left(E = \frac{\Delta \text{ tension} \times \text{length}}{\Delta \text{ length} \times \text{cross section}} \right),$$

i. e. the force (in dynes) which must act upon a cross section of 1 sq.cm. of the body in order to double its length ($\Delta \text{ length} = \text{length}$). It is, however, not only the longitudinal elasticity but also the torsional elasticity, which is used to define the elastic properties of a body. The present investigation deals exclusively with longitudinal elasticity.

When examining the mechanical properties of muscle fibres, it is not so much the absolute values which are of importance, but rather the relative changes which occur when

muscle fibres are stretched or contracted. By this method it is possible to obtain information of the minute structure of the fibres and of the changes which occur in the latter during extension and contraction.

Muscle fibres belong to the so-called high-elastic substances, in which a slight variation of tension will produce a considerable change in length and cross section. When the variation of the minute structure of the muscle fibres is just the aim of the investigation, it is most convenient to use the stiffness, i. e. the ratio of corresponding increases of tension and length (Δ tension/ Δ length), as emphasized by *Buchthal* (1942, p. 67). The stiffness may be recorded directly without it being necessary to determine the cross section of the muscle fibres. The variation of stiffness with tension is primarily caused by changes in the molecular and micellar minute structure of the muscle fibres and, to a smaller extent, by the inversely proportionate changes in length and cross section.

By the viscosity of muscle fibres we understand the internal resistance (damping) and the elastic after-effects of more or less rapid variations in length which result in deficient stabilization. In static experiments, where only stabilized values are examined, the viscous damping and elastic after-effects have ceased. In semi-dynamic and dynamic experiments the length-tension variation is recorded while the viscous properties are active or before they have begun manifesting themselves; the more rapid the variation in length the less the viscosity will reduce the variation in tension. As the muscle fibres possess free elasticity as well as elasticity damped by viscosity, static experiments will serve to determine the total elasticity, while dynamic experiments will essentially furnish a measurement of the free — not damped — elasticity.

When examining the dynamic stiffness by means of periodical variations in length the stiffness is thus found to vary with the frequency (*Buchthal, Kaiser & Knappeis*, 1944). For the determination of the stiffness of mammalian muscle fibres the frequencies of the variations in length employed have been 10 and 100 cycles per sec.

Finally the muscle fibres have plastic properties, as mechanical stresses exceeding a certain magnitude may produce small irreversible deformations of the muscle fibres, e. g. elongation of the equilibrium length, which are not tantamount to lesions of the structure. Yielding during contraction, which

is dealt with in a later section, is, for instance, a plastic function.

It applies equally much to the following investigations as to the quantitative birefringence determinations that the experiments should be performed on individual muscle fibres, or at any rate on small bundles with parallel muscle fibres of uniform length from which the fascia has been removed. When investigating whole muscles there are too many uncontrollable and variable factors caused by differing lengths and dimensions of fibres, varying course of fibres and heterogeneous contents of connective and tendinous tissue. It is only during the last ten years that the mechanical properties of muscle fibres have been examined by means of isolated fibres and fibre bundles (*Sichel*, 1934; *Asmussen*, 1934, 1936; *Ramsey & Street*, 1940; *Buchthal*, 1942; *Buchthal & Knappeis*, 1943, a and b; *Lundin*, 1944; *Buchthal, Kaiser & Knappeis*, 1944, and *Buchthal & Kaiser*, 1944). All previous experiments right from *E. Weber's* investigation in 1846 deal with the mechanical properties of whole muscles.

The investigations on the mechanical properties of mammalian muscles are chiefly made for the purpose of comparing them to the experiments on the fibres of the frog, and are consequently most conveniently described in conjunction with the latter.

A. Static Experiments.

Static Length-Tension Diagrams during Rest.

Fig. 43 shows the mean curve of the length-tension diagrams of nine different fibre bundles. Length-tension diagrams of fibres from *m. gluteus max.* and *m. serratus post.* show no fundamental difference. In the beginning the increase of the tension in relation to the elongation is only small, i. e. the stiffness increases only slightly; it is not until an elongation of 30—40 per cent has been reached that the tension increases relatively more than the length, i. e. the static stiffness increases. In a few experiments the fibres have been stretched up to 100 per cent (fig. 44), and in all experiments the static stiffness is found to increase with increasing stretch.

The small increase of tension at low degrees of stretch may probably be explained by the fact that the micellae are not completely longitudinally orientated at equilibrium length of the fibres — as shown by *Buchthal & Knappeis* (1940) by their investigations on the diffraction spectra of muscle fibres — but become straightened out during stretch. When fibre length exceeds 110—130 the micellae are completely straightened

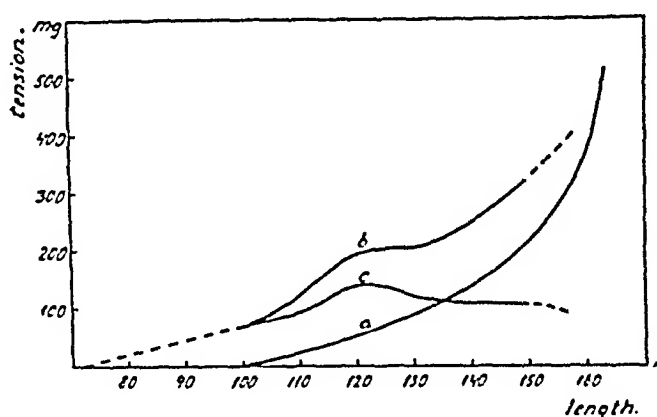


Fig. 43.

Static length-tension diagram of muscle fibres at rest and during isometric, tetanic contraction.

a) tension at rest. b) total tension (= tension at rest + extra-tension during contraction). c) extra-tension during contraction for isometric, tetanic contraction.

a) mean curve of 9 experiments. b) and c) mean curves of 5 experiments on fibre bundles from m. gluteus max. and m. serratus post. (conf. text p. 154).

Abscissa: length of fibre (equilibrium length = 100).

Ordinate: tension in mg.

out, consequently the curves do not until then supply a true picture of the elastic conditions of the minute structural elements. *Sichel* (1934—1935) observed the length-tension diagrams of isolated muscle fibres to be linear at low degrees of stretch, while the curves for whole muscles showed increasing static stiffness, also at low degrees of stretch. *Asmussen* (1936) found the length-tension diagrams to be uniform for individual muscle fibres and for fibre bundles and both he and *Buchthal* stated the first part of the length-tension graph to be curved with the concavity upwards. The corresponding diagrams of cardiac muscles (*Lundin*, 1944, p. 40), on the other hand, show a steeper gradient at the beginning — the concavity of the curve facing downwards — a similar length-

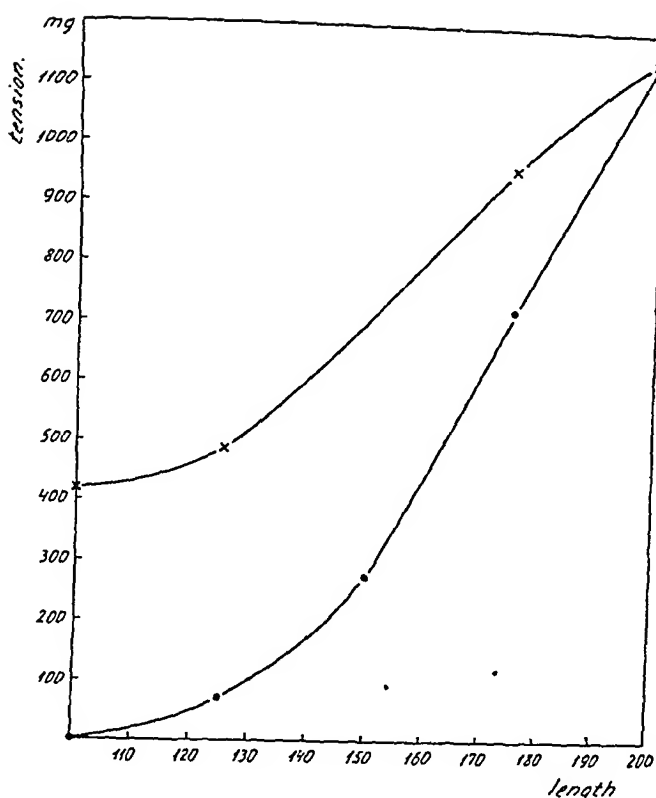


Fig. 44.

Static length-tension diagram of muscle fibres at rest (•) and during isometric, tetanic contraction (X). Single experiment on a fibre bundle from *m. serratus post.*

Abscissa: length of fibre (equilibrium length = 100).

Ordinate: tension in mg.

tension curve as exhibited by rubber. The minute structural elements of the cardiac muscles are less pre-orientated at equilibrium length than those of skeletal muscles.

Ramsey & Street (1940) claim that the length-tension diagram is exclusively determined by the sarcolemma, as they find identically shaped curves for intact muscle fibres and for muscle fibres with damaged contents, but with intact sarcolemma. In contrast with this *Sichel* (1941) has later on shown that the stiffness of the sarcolemma of damaged parts of muscle fibres is one to four times less than that of intact fibres. In damaged fibres the sarcolemma will moreover be deformed, which must result in an increase of its "normal" stiffness. The sarcolemma only amounts to a small part of the volume of the muscle fibres, also for this reason it is unlikely

that the length-tension diagram of the muscle fibres should be caused by the elasticity of the sarcolemma.

The static length-tension diagram of muscle fibres at rest is only reversible for extension up to length 140—150, and the equilibrium length is not a completely constant value either.

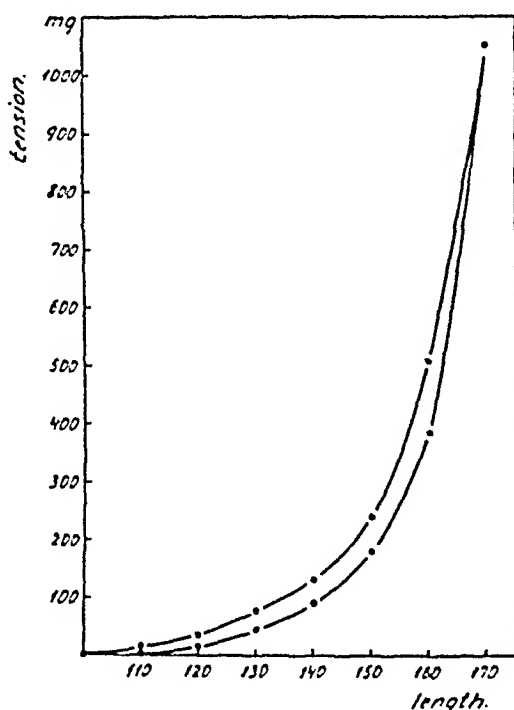


Fig. 45.

Static length-tension diagram of resting muscle fibres at stretch (top curve) and release (bottom curve). Single experiment on fibre bundle from *m. serratus post.*

Abscissa: length of fibre (equilibrium length = 100).

Ordinate: tension in mg.

At higher degrees of stretch — above length 145 in frog fibres, according to *Asmussen* (1936) — an elongation of the equilibrium length of the muscle fibres will occur on account of a low degree of plasticity. Thus the muscle fibre is not an ideal elastic body. Fig. 45 represents an irreversible, static length-tension diagram from mammalian muscle fibres illustrating the fact that the equilibrium length has increased by about 10 per cent after the fibres have been stretched to length 170.

Static Length-Tension Diagrams during Tetanic Contraction.

The absolute increase of tension during isometric contraction of course differs widely in the various fibre bundles, but the relative increase of tension during contraction, calculated in per cent of the tension at rest, also varies considerably from preparation to preparation. A mean curve of five experiments (fig. 43), in which the increase of tension has thus been calculated in per cent of the tension at rest, shows that the tension during contraction at equilibrium length corresponds to a tension of the resting fibres at length 125. In some preparations the extra-tension during contraction (fig. 43, curve c = the difference between curves a and b) has the same value at equilibrium length and at elongations up to length 150, while other preparations show higher extra-tension during contraction at length 120—130 than at equilibrium length — this also applies to the mean curve (fig. 43). Such a maximum on the curve of the extra-tension during contraction might be due to different degrees of stretch of the individual fibres of the bundle, but it may, however, also be found in curves representing isolated muscle fibres. *Asmussen* (1936) f. inst. examined frog fibres which had their largest extra-tension during contraction at length 120—130. A slight extension of the fibres thus seems to provide optimum conditions for a good contraction — possibly on account of the complete longitudinal orientation of the micellae.

In case of extension beyond length 140—160 the extra-tension during contraction decreases and finally becomes nil — just as in frog fibres (shown e. g. already by *Blix*, 1895). According to *Buchthal* this condition occurs in frog fibres when the fibre has been stretched 60—100 per cent. A few experiments at such high degrees of stretch are carried through with mammalian muscle fibres, and the conditions observed are the same. Decrease of the extra-tension during contraction with increasing extension may have the results that the total tension during contraction also decreases with increasing length at the higher degrees of stretch. In that case the shape of the curve b will not be regularly concave, but will exhibit a small peak.

The reason why the extra-tension during contraction decreases at high elongations was assumed by *Asmussen* (1936) to be a decreasing irritability, but later experiments by *Buch-*

thal (1942, p. 22) have shown that it is mainly due to a reduced contractility — a fact which is related to the above-mentioned “yielding” — a plastic elongation of the equilibrium length of the fibres during contraction, which will occur when the contracted fibres are exposed to tension beyond a certain magnitude. “Yielding” is dealt with in details below.

Curve b of fig. 43 represents the maximum tension during isometric contraction, but the values of the maximum tension as a function of length are not reversible — as already shown by *v. Kries* (1880) and *Blix* (1892). If a fibre is released during contraction the tension will decrease according to a curve the gradient of which is steeper than that of the “isometric maxima”, as shown i. a. by *Sulzer* (1930) and *Asmussen* (1936). According to *Buchthal* (1942, p. 24) this fall in tension is chiefly due to an elastic locking of the contracted fibres — not to a defective stabilization (viscosity) as asserted by *Gasser & Hill* (1924). It was shown by *Buchthal* that when an isometrically contracted fibre during continuous contraction is released to the same tension as at rest, a considerably lower tension will be reached than the one corresponding to the tension during isometric contraction at this new length — in spite of the fact that the fibre is allowed to stabilize completely after the release. At the maximum of the isometric tetanic contraction the fibre has become elastically locked at its contraction length.

On the cardiac muscles of the frog *Lundin* (1944, p. 43) could not observe any locking during contraction, as release produced a fall in tension following curves with the same gradient whether the cardiac muscle fibres were contracted or at rest.

By stretching contracted muscle fibres a tension is produced, which is above the curve of the isometric maxima (*Beck*, 1923; *Sulzer*, 1930; *Asmussen*, 1936), at any rate for the degrees of stretch above length 150 (*Buchthal*, 1942, p. 22). *Asmussen* considers this to indicate incomplete stabilization, while *Buchthal*, 1942, p. 22) also found this condition to prevail in cases where full stabilization must have been reached, and explains the condition on the basis of his model of the minute structure (conf. p. 181).

In this connection it is of interest to ascertain the shortest length the muscle fibres may attain during contraction, i. e. the contraction equilibrium length. As a consequence of the

elastic locking this is no uniform figure, but depends on the external conditions of the contraction. In case of tensionless contraction the shortening will be more pronounced, than if the fibres are first allowed to shorten during contraction and then released to tension zero. The length of contracted fibre bundles released from isometric contraction to tension nil has been determined on two different preparations, the length, tension and stiffness being recorded simultaneously while the fibres are stretched and released four to five times during continuous contraction. Tension zero is determined both directly and by means of the stiffness. The results of the experiments show that by tension nil the length of the fibres at release-contraction is 64—70 to 74—80, i. e. on an average 72 (equilibrium length 100). The values are found to be slightly lower when determined during stretch, namely 64—70, than when determined during release, namely 74—80, which is due to defective stabilization (viscosity) of the fibres, the experiments in question being semi-dynamic experiments.

On isolated frog fibres *Buchthal* (1942, p. 24) found the contraction equilibrium length at release-contraction to be 55 (45—65). When using fibre bundles it is of special importance in these experiments, that the fibres of the bundle have the same length and are stretched to the same extent. If a few of the fibres of the bundle are stretched at the rest equilibrium length found for the whole bundle, these fibres may during release-contraction produce a curving of the other fibres, and the shortening of the latter fibres during contraction is measured to be greater than it actually is. These circumstances explain why the shortening during contraction in experiments on whole muscles has been found to be more pronounced — up to 85—90 per cent (*Weber*, 1846) — than in experiments on isolated fibres or small fibre bundles of homogeneous structure. On isolated fibres of the frog *Ramsey & Street* (1940) found a shortening during contraction of up to 65—70 per cent of the "equilibrium length". As equilibrium length they employed the length at which the extra-tension during contraction was maximum; this corresponds to length 125 according to my definition, so that correspondingly the contraction equilibrium length will be 38—44. Their experiments do not, however, deal with reversible contractions; they themselves say, that after such a maximum contraction the fibres pass into a state referred to by them as "delta state" and now

exhibit a permanent shortening of the equilibrium length and an altered length-tension diagram during contraction. *Ramsey & Street's* microphotographs of such fibres moreover show a destroyed cross striation. Therefore this kind of contraction can hardly be considered as physiological, but it is due to lesions of the fibres.

Length-Tension Diagrams for Single Contractions.

Figs. 46 and 47 show examples of tension variations during isometric single contractions as a function of time, recorded at different degrees of stretch. Only the extra-tension has been measured. The initial tension at the different degrees of stretch is neglected. The experiments show that the increase of the tension during single contraction is highest at fibre length 115—125, while it is smaller at equilibrium length and decreases in case of further extension of the fibres. This observation corresponds to the conditions of isometric, tetanic contraction (fig. 43) and has also been ascertained for fibres of the frog (*Asmussen*, 1934, 1936; *Buchthal*, 1942, p. 29, 1944). In his first investigation on the mechanical properties of the muscle fibres (1942, p. 33) *Buchthal* claims, that the peak tension in case of single contraction is reached in the course of the same time, irrespective of the degree of stretch of the fibres, while the descending part of the curve is longer the higher the degree of stretch of the fibres, only in case of fatigue the ascending part of the curve is found to be of varying length. In my experiments the duration of the actual contraction phase is shortest at equilibrium length, but longer the higher the degree of stretch of the fibres, and this in spite of the fact that the order in which the experiments have been performed does not follow the degree of stretch. The preparation of fig. 46 was f. inst. first subjected to contraction at length 118, then at lengths 109, 145 and finally at length 127. It may be mentioned that the temperature of the muscle chamber has been controlled continuously during these experiments. In experiments where mammalian muscle fibres have been exhausted, a fact which manifests itself through a decrease of the peak extra-tension during contraction, no significant increase of the duration of the contraction has been observed (fig. 48). *Buchthal & Kaiser's* later experiments

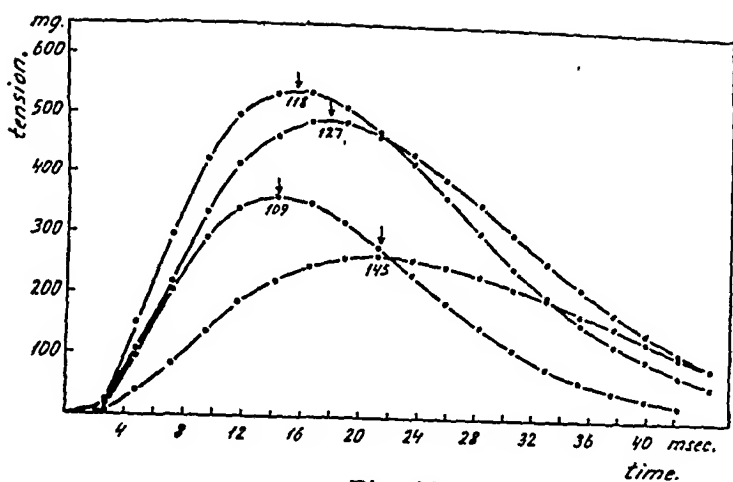


Fig. 46.

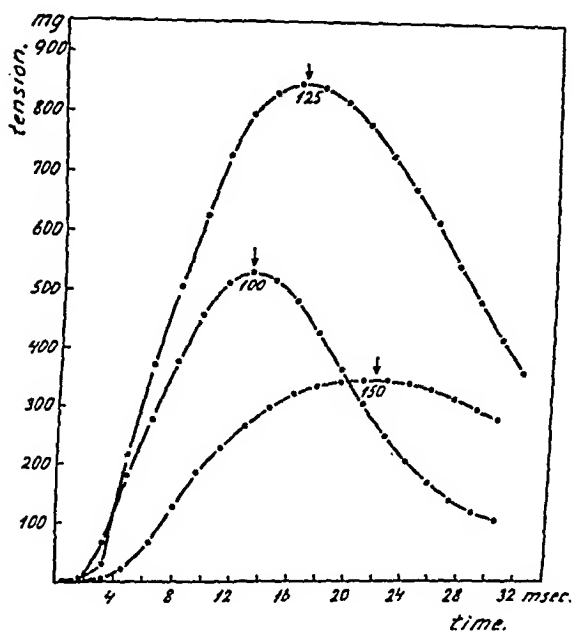


Fig. 47.

Figs. 46—47.

Variation of tension during isometric, single contraction as a function of time.

Experiments at various degrees of stretch of the same fibre bundle. The figures below the curves represent the lengths of the fibres. The arrows indicate the maxima of the curves.

Preparation: in both experiments fibre bundle from *m. gluteus max.*

Abscissa: time in msec.

Ordinate: tension in mg.

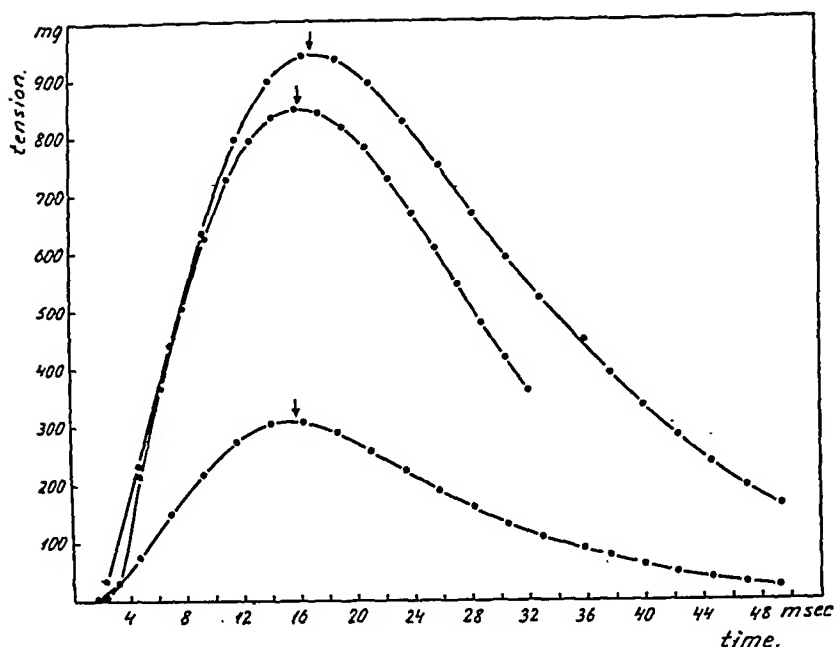


Fig. 48.

Variation of tension during isometric single contraction in case of fatigue. Experiments at various states of fatigue of the same fibre bundle.

The arrows indicate the maxima of the curves.

Preparation: fibre bundle from m. gluteus max.

Abscissa: time in msec.

Ordinate: tension in mg.

(1944) also showed interdependence between the degree of stretch of the fibres and the duration of the contraction phase.

As regards mammalian muscle fibres the duration of the single contractions is 35—45 msec., while the actual contraction phase lasts for 15 msec., this figure being recorded at a temperature of 37—38°C. According to *Buchthal* (1942, p. 33) the single contraction lasts 100—150 msec., and the contraction phase 40—50 msec. in muscle fibres of the frog examined at 18—20°C.

Length-Tension Diagrams for Single Contractions, Repeated at Varying Intervals.

If the muscle fibres are stimulated by stimuli with gradually increasing and decreasing frequencies, it is possible to study the manner in which a tetanic contraction is built up, figs. 49—50. Fig. 50 shows the results of an experiment

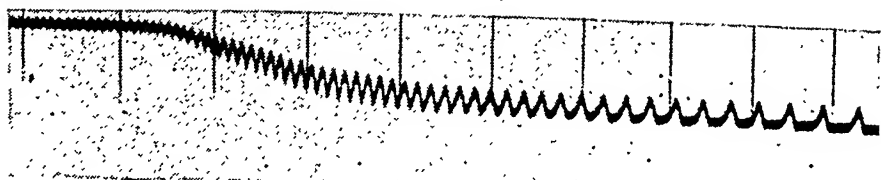


Fig. 49.

Building up a tetanic contraction. Record of the variation of tension during isometric contraction of a fibre bundle which is stimulated by single impulses with increasing frequency: 2—100 stimuli per sec. (Time marked for every $\frac{1}{16}$ sec.). Curve recorded from the right to the left.

Preparation: fibre bundle from m. serratus post.

carried out at equilibrium length of the muscle fibre. The curve is a mean curve of the ascending and descending values — corresponding to increasing and decreasing frequencies. In this way the errors are equalized which might be caused by defective stabilization, occurring if the variation of the frequency takes place so rapidly that the fibres cannot “keep up with” the frequencies of the impulses. In the experiment

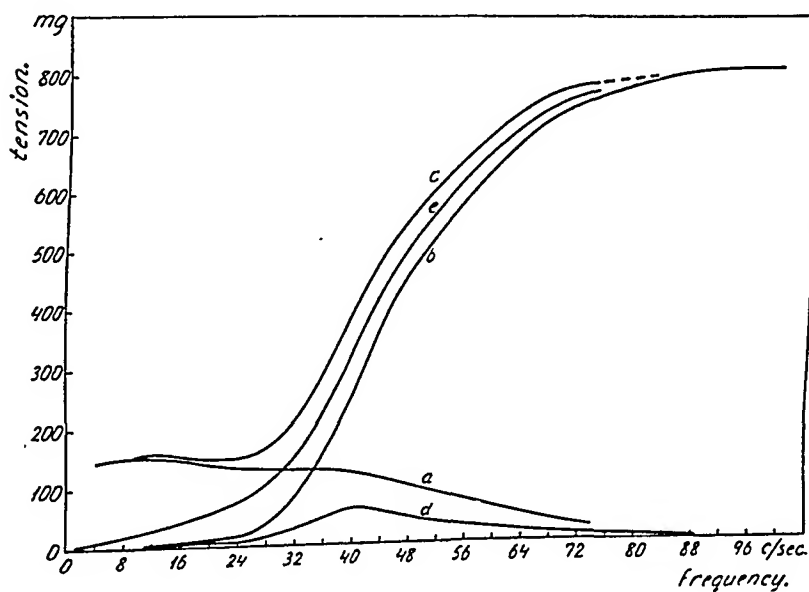


Fig. 50.

Variation of tension during isometric contraction of a fibre bundle at equilibrium length as a function of the frequency of stimulation.

a) extra-tension during single contraction. b) remaining tension. c) total tension (= remaining tension + extra-tension at single contraction). d) increase of remaining tension per single contraction. e) mean tension.

The curve is a mean curve of ascending and descending values. (conf. text p. 160). Abscissa: frequency of stimuli per sec.

Ordinate: tension in mg.

represented the extra-tension during contraction (i. e. the difference between total tension during contraction and the remaining tension) is constant during a frequency increase from 4 to 16, then it decreases very slightly, but it only decreases perceptibly at impulse frequencies of more than 40 cycles per sec., at frequencies of 85—90 the contraction is purely tetanic, i. e. the tension is constant. Already at a frequency of 10 a remaining tension is developed, after the single contractions the tension do not fall to the same value as before, a summation takes place. At frequencies about 25—30 the single contractions follow each other without any interval. This corresponds to a duration of the single contraction of 35—40 msec. The mean tension, which is an expression of the total mechanical performance of the fibre bundle, here reaches a value half way between the resting or remaining tension and the total tension — the mean tension being calculated on the basis of the frequency-tension diagram. When the stimulation frequency is increased from 30 to 70 cycles per sec. the remaining tension rises sharply. At frequencies of 85—90 remaining tension and total tension coincide. The peak tension in case of single contraction at frequencies below 16 cycles per sec. amounts to one fifth of the total tension in case of tetanus.

The experiment represented in fig. 50 showed, as mentioned, a tetanus at equilibrium length of the fibres. The curve is modified at the different degrees of stretch, the extra-tension during contraction being higher at fibre length 115—125 than at equilibrium length, but decreases when the extension is further increased. Fig. 51 shows the variations of the length-tension diagrams with varying frequency of the stimulation, it will i. a. appear from this figure that it is only at frequencies above 70—80 cycles per sec. that the contraction tension is stabilized corresponding to a complete tetanus.

The above mentioned results conform on the whole to the results of the experiments on frog fibres (*Buchthal*, 1942, pp. 30—33, fig. 14) with the modification caused by the longer duration of the single contractions of frog muscles. *Buchthal* found: the resting tension begins to increase at a frequency between 5 and 10 cycles per sec.; the extra-tension during contraction reaches its maximum value at frequencies about 12 and then decreases. Already at impulse frequencies of 30—40 cycles per sec. the contraction of the frog fibres is

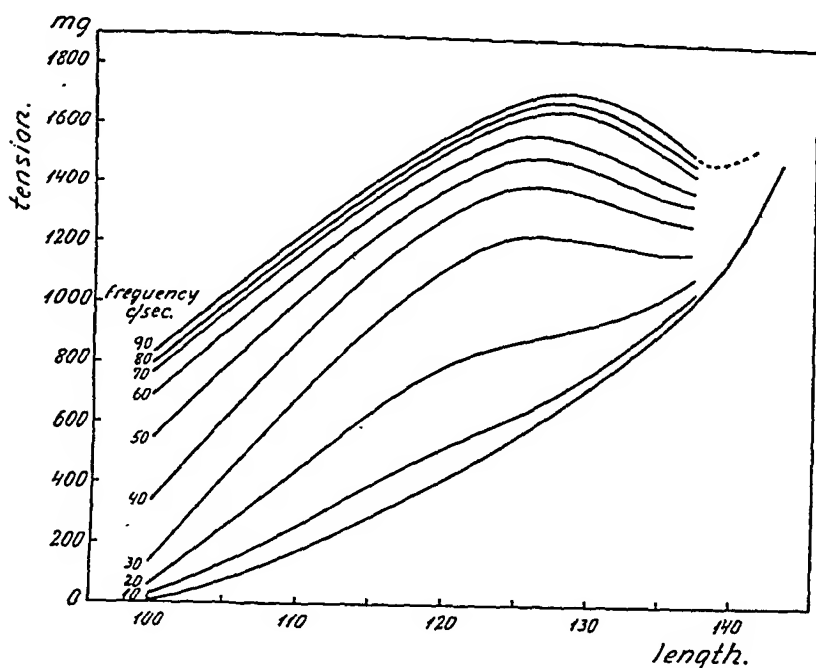


Fig. 51.

Relation of length to mean tension at various frequencies of stimuli. Experiments on a single fibre bundle from *m. gluteus max.* The figures represent frequency of stimuli per sec. Abscissa: length of fibre (equilibrium length = 100). Ordinate: tension in mg.

fully tetanic. The duration of the single contraction amounts to approximately 100—150 msec. and the extra-tension at single contractions amounts on an average to one fifth of the tension at full tetanus.

The Absolute Extra-Tension during Contraction of the Muscle Fibres.

The absolute extra-tension during tetanic contraction is determined in the experiments on mammalian fibres to be 8—10 mg. per muscle fibre. *Clark* (1931) calculated the corresponding value for muscle fibres from cats to be 40—90 mg. *Buchthal* (1942, p. 45) found an increase of the tension of 10 mg. during contraction of isolated muscle fibres of the frog. According to *Eccles & Sherrington* (1930) the individual motor unit of a cat's muscles can yield a tension of 5—30 g. during contraction. If the extra-tension during contraction of 150—300 fibres — i. e. the number of fibres of which a motor

unit is stated to consist (conf. *Clark*, 1931) — is calculated on the basis of the present experiments, the result will be an extra-tension during contraction of 1—3 g. for each motor unit.

B. Semi-Dynamic Experiments.

In the previous section it has been shown that the muscle fibres are elastic and plastic, the latter only to a slight extent, however. Through dynamic experiments the elasticity of the muscle fibres is further to be elucidated, and it will be shown that the muscle fibres also possess viscosity. The semi-dynamic experiments will be dealt with first; in these experiments stretching and relaxation occur, as mentioned, at a limited speed, i. e. they last from 1—10 sec.

Semi-dynamic length-tension diagrams have been investigated by *v. Kries* (1890); *Blix* (1892); *Fick* (1892) and later on by *Sulzer* (1930). Semi-dynamic experiments have also been performed by *Gasser & Hill* (1924) and by *Levin & Wyman* (1927), but they chiefly used another experimental technique for illustrating the viscous-elastic properties of muscle fibres. All the investigators mentioned have used whole muscles as test objects, *Buchthal* (1942) being one of the first to use isolated muscle fibres. However, whether whole muscles or isolated muscle fibres of the frog are used, the length-tension diagram of resting fibres during stretch is found to differ from that of relaxation, the tension being lower during relaxation than during stretch, if measured at the same length. This is caused by the fact that muscle fibres possess a viscous resistance against variations in length.

Mammalian muscle fibres also exhibit defective stabilization in semi-dynamic experiments which shows them to be viscous. Continuous stretch and relaxation of a resting fibre bundle with gradually increasing amplitude may be stated as an example: At the beginning of the experiment the bundle at length 9.2 mm. displayed a tension of 40 mg., after 22 per cent stretch and relaxation to length 9.2 mm. the tension was 25 mg.; after 33 per cent stretch and renewed relaxation to 9.2 mm. the tension was 25 mg.

The viscosity of the muscle fibres has the effect that the equilibrium length of the fibres becomes longer, but this

elongation is reversible in contrast with the above mentioned moderate plastic elongation which does not occur until the fibres have been stretched more than 45 per cent (Asmussen, 1936). The time which passes before the fibres are completely stabilized will i. a. depend on the rate and size of the variation in length.

Semi-dynamic length-tension diagrams from contracted muscle fibres show that the fibres also possess viscosity during contraction, but the most characteristic feature of these

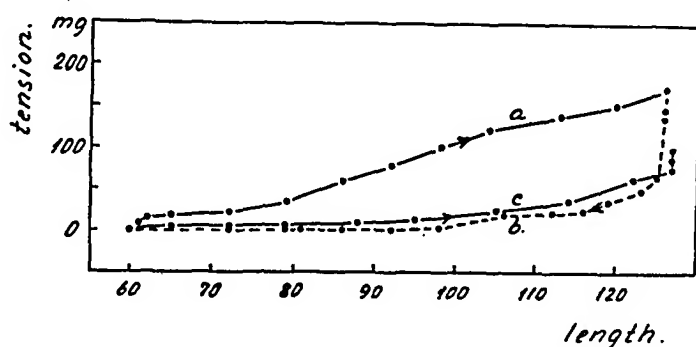


Fig. 52.

Semi-dynamic length-tension diagram of contracted fibre bundle. The fibres contracted in a tensionless state, stretched (a), released (b) and again stretched (c) during continuous contraction.

Preparation: fibre bundle from *m. gluteus max.*

Abscissa: length of fibre (equilibrium length = 100).

Ordinate: tension in mg.

diagrams is the plastic yielding which occurs when contracted fibres are exposed to a certain tension, and the elastic locking which causes the fibres during continuous contraction to maintain the contraction equilibrium length even when they are passively released. *Buchthal* (1942, pp. 38—43) used semi-dynamic experiments to demonstrate these phenomena on skeletal muscles of the frog, and in fig. 52 an example of corresponding yielding and locking of contracted mammalian muscle fibres is shown. In the cardiac muscles of the frog *Lundin* (1944, pp. 41—43) could not, however, ascertain any yielding or locking.

In the experiment (fig. 52) the mammalian muscle fibres are contracted in a tensionless state, during continuous contraction stretched to length 120 (curve a), released to length 55 (curve b) and again stretched to length 120 (curve c). The difference between the two last mentioned curves (b and c)

is due to viscosity. The difference between a and b is explained by an elongation of the equilibrium length of the fibres during extension — a “yielding”, and as the elongation is irreversible as long as the contraction lasts, the contracted fibres must also be elastically locked. The difference is larger than the plastic elongation which may be observed on stretching resting fibres, and the latter does only occur when the extension exceeds length 145.

C. Dynamic Experiments.

The reactions of the muscle fibres have been studied with regard both to torsional movements and to vibrations along the longitudinal axis of the fibre. The structural properties investigated by these two methods are different; this, of course, applies especially to the muscle fibres which possess an inhomogeneous structure with alternate isotropic and anisotropic substances, but it should be noted that simple crystals also have several different moduli of elasticity. The torsional elasticity of muscle fibres of the frog has been investigated i. a. by *Lindhard & Møller* (1926, 1928).

As mentioned before, the dynamic longitudinal elasticity of mammalian muscle fibres has been measured by exposing the fibres to periodical variations in length and has been expressed as the stiffness ($= \Delta \text{ tension} / \Delta \text{ length}$). Stiffness is the most convenient measurement of the longitudinal elasticity of the muscle fibres, and moreover it is simple to determine by the experimental technique used — the periodical variations in length and corresponding variations in tension being recorded directly.

Gasser & Hill (1924) calculated the dynamic longitudinal elasticity of frog muscles on the basis of the change produced in the natural vibration of a vibrating system, when the vibrations of the system are damped by the resistance offered by a straightened-out frog muscle attached to it. They also investigated the tension reaction of the muscle in case of more or less rapid extension and release and found that the muscle becomes less extensible and more viscous during isometric contraction. *Buchthal* (1942, p. 72) has later on shown that the stiffness is increased with increasing tension, a fact of

which *Gasser & Hill* have not been aware. For this reason it is impossible to make any deductions from their experiments, as to whether the increase of the viscous-elastic properties during contraction is solely due to the simultaneous increase in tension, or whether it is also caused by an altered structural condition.

Steinhausen (1926) and, later on, *Richter* (1928) have measured the period of oscillation and the damping when a frog muscle is exposed to sudden extensions by means of a modified *Bethe's* (1924) elastometer. They compensated for tension, and they did not find any change in the modulus of elasticity of the frog muscle in case of isotonic tetanus; in case of isometric tetanus the modulus of elasticity is on the other hand altered, but only to an extent corresponding to the increase in tension — a passive increase in tension produces an alteration of the elasticity of the same magnitude.

Levin & Wyman (1927) have investigated the length-tension diagrams of various muscles performing the stretch and release at different rates. By comparing the dynamic stiffness to the dynamic after-effect they could set up an equivalent of the viscous-elastic properties of the muscle (fig. 53). They

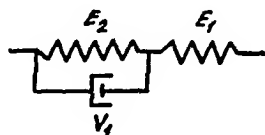


Fig. 53.

Equivalent of the viscous-elastic function of muscle fibres (according to *Levin & Wyman*, 1927). E_1 : free elasticity, E_2 : elasticity damped by viscosity (V_1).

stated that the muscle possesses both free elasticity and elasticity damped by viscosity. In contrast with this *Gasser & Hill* (1924) have stated that all the elasticity of the muscle is "shunted" by viscosity. *Bouckaert et al.* (1930) have, however, confirmed *Levin & Wyman's* hypothesis, and in later investigations (i. a. 1939) *Hill* also goes in for the theory that the muscle has both free and damped elasticity.

It should be remembered that all the above mentioned investigations have been performed on whole muscles. *Buchthal et al.* (1942—1944) have, however, carried out comprehensive investigations on stiffness on isolated fibres of the frog and have e. g. investigated stiffness as a function of ten-

sion, of frequency and of temperature, and an account of the latter investigation will be given together with the description of the orientating experiments on mammalian muscle fibres performed by the author within this field.

Buchthal (1942, p. 72) found that the dynamic stiffness of the fibres from frogs varies proportionately with the tension

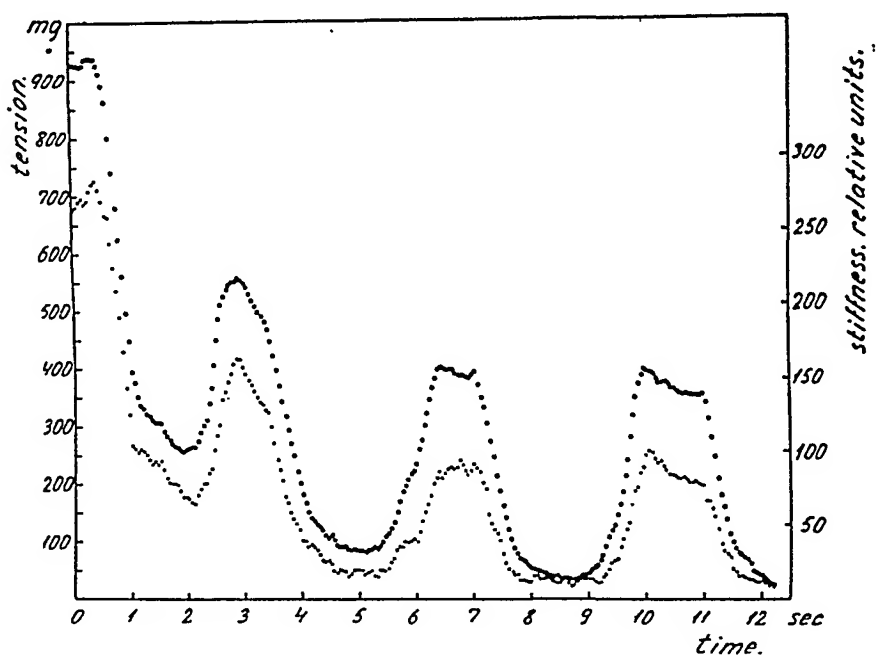


Fig. 54.

Variation of tension (···) and stiffness (- · - ·) as a function of time.

Resting fibre bundle from *m. serratus post.*, continuously released and stretched from length 144—133—144—122—144—111—144—100.

Abscissa: time in sec.

Ordinate: (to the left) tension in mg.

(to the right) stiffness (determined at 10 cycles per sec.) in relative units.

— except at very low degrees of stretch at which the stiffness may be constant in spite of slight increase of tension. Figs 54—55 show that similar stiffness-tension diagrams are obtained for mammalian muscle fibres at rest; the stiffness is here determined for a frequency of 10 cycles per sec. Figs. 56—59 actually illustrates the same thing; in figs. 58—59 the stiffness is determined for a frequency of 100 cycles per sec. Already at tension nil a slight stiffness is observed. This indicates that the fibres have a certain structure even when they are without load, and it appears because a temporary length-tension

increase — corresponding to half of the vibration amplitude — is imparted to the fibres at equilibrium length.

When constant stiffness is observed at low degrees of stretch in spite of increasing tension, this may be caused by the micellae not being completely longitudinally orientated at equilibrium length of the fibres (conf. the investigations on diffraction spectra). It should be noted that the stiffness increases linearly with increasing tension, which means that the elements of the minute structure are completely longitudinally orientated. In this respect the muscle fibres differ from other

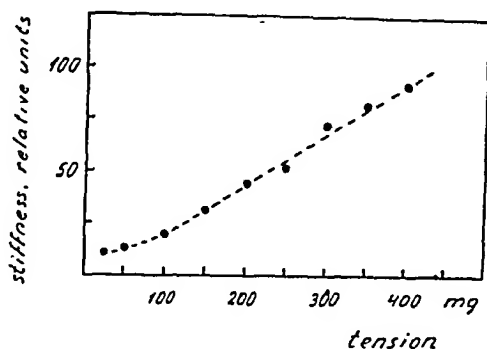


Fig. 55.

Stiffness of resting fibres as a function of tension.

Mean values from the experiment in fig. 54.

Preparation: fibre bundle from *m. serratus post.* at length 100–144.

Abscissa: tension in mg.

Ordinate: stiffness (determined at 10 cycles per sec.) in relative units.

high-elastic substances, e. g. rubber, which do not exhibit a linear stiffness-tension diagram until it is stretched 400 per cent (*Buchthal*, 1942, p. 120); in rubber the minute structure does not become longitudinally orientated until the degree of stretch exceeds 400 per cent.

In his investigation (1942, pp. 87–88) *Buchthal* showed that the ratio of static to dynamic stiffness measured at 5 vibrations per sec. is as 1 to 2 and only varies slightly when the fibres are stretched. This ratio shows that only part of the elasticity of the muscle fibres is damped by viscosity and indicates that the viscosity of the muscle fibres is not uniformly distributed over the elastic elements. In the cardiac muscles of the frog the ratio of static to dynamic stiffness at rest is as 1 to 10–16 (*Lundin*, 1944, pp. 56–57), here the viscosity is of more importance than in skeletal muscles. By examining the stiffness at various frequencies *Buchthal*, *Kaiser* & *Knap-*

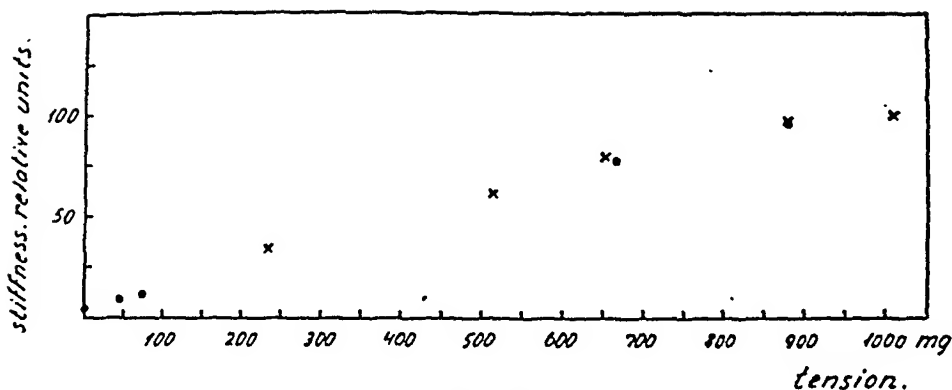


Fig. 56.

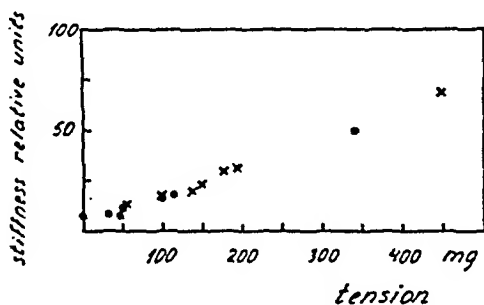


Fig. 57.

Figs. 56—57.

Stiffness during rest (•) and during contraction (×) as a function of tension. 2 single experiments.

Preparations: fibre bundles from m. gluteus max. at length 100—200 (fig. 56) and 100—156 (fig. 57).

Abscissa: tension in mg.

Ordinate: stiffness (determined at 10 cycles per sec.) in relative units.

peis (1944) found that the more rapid the frequency used the greater the stiffness and the steeper the gradient of the stiffness-tension curve exhibited by the skeletal muscle fibres of the frog. The relation between stiffness and tension of the muscle fibres reveals a dependence upon the frequency corresponding to a system of elasticity "shunted" by viscosity. The plastic and viscous properties are most conveniently examined by means of low frequencies, at high frequencies, however, the purest expression of the actual elastic properties is obtained, a frequency of 100 cycles per sec. is e. g. very convenient for the latter purpose.

Buchthal, Kaiser & Knappeis (1944) did not observe any

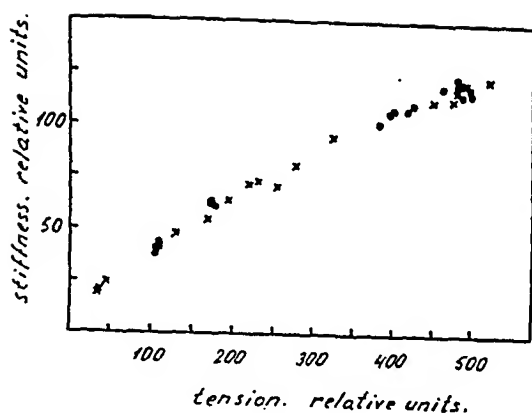


Fig. 58.

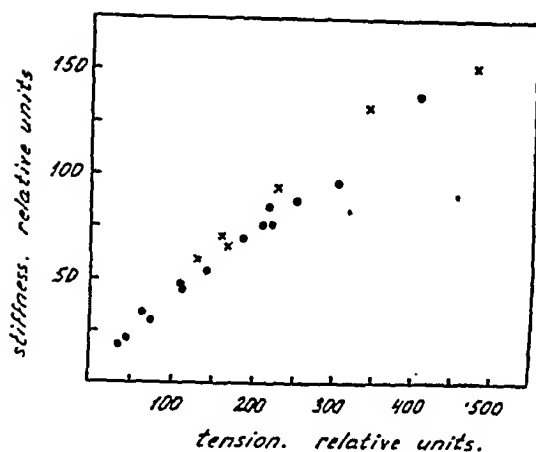


Fig. 59.

Figs. 58—59.

Stiffness during rest (•) and during contraction (×) as a function of tension, 2 single experiments.

Preparation: fibre bundles from *m. gluteus max.* at length 100—156 (fig. 58) and length 100—167 (fig. 59).

Abscissa: tension in relative units.

Ordinate: stiffness (determined at 100 cycles per sec.) in relative units.

difference in the stiffness of resting fibres of the frog whether examined at a temperature of 4° or 24°C. This is strange, in so far as a dependence between stiffness and temperature should be expected to exist in a viscous system.

To elucidate the function of the minute structure the conditions during contraction should be examined or — more correctly — the conditions during rest and contraction should be compared. In view of the experimental technique available

it was most convenient to use isometric contractions for the comparison of stiffness at rest and stiffness during contraction. In isometric contraction the length is kept constant while the tension is varied. Since tension influences stiffness, whether it is caused by passive stretching of the muscle fibres or by actual contraction, the stiffness at rest and during contraction should only be compared when referred to the same tension, when the purpose is to explain the influence of the actual contraction process on the variation of the stiffness.

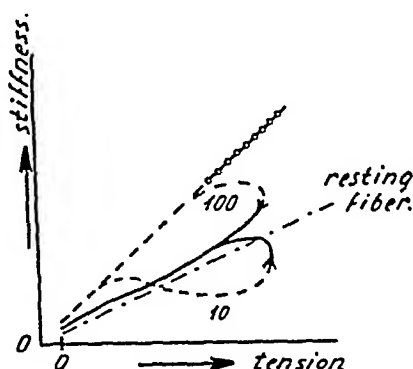


Fig. 60.

Schematic diagram of stiffness as a function of tension during stretch and release of contracted frog muscle fibres (according to *Buchthal, Kaiser & Knappeis, 1944*).

The stiffness is examined for 10 and for 100 cycles per sec. The arrows indicate the sequence of the values. (The fact that the stiffness is higher when determined at 100 cycles per sec. than at 10 cycles per sec. even during release has not been taken into consideration in this diagram).

— . — . — rest. — — — extension during contraction. — — — release during contraction. —○— relation of stiffness to tension determined at 100 cycles per sec., if no yielding occurred.

In muscle fibres of the frog (*Buchthal, Kaiser & Knappeis, 1944*) the relationship between stiffness and tension during contraction is rather complicated, i. a. on account of yielding. This is best illustrated by a schematic diagram (fig. 60, according to *Buchthal, Kaiser & Knappeis, 1944*). When examining the stiffness for periodic length variations of a frequency of 100 cycles per sec. the actual vibrations cannot produce yielding. Consequently the dynamic stiffness is found to increase linearly with the tension for isometrically contracted fibres as well as for resting fibres, but during contraction the stiffness is higher than at rest when referred to the same tension, and this applies to extension as well as to release. The

stiffness-tension diagram during contraction exhibits a steeper gradient for values obtained during stretch than for those obtained during release. This is conditioned by the yielding, which is caused by the actual extension, and by the elastic locking, due to which the fibres maintain the higher equilibrium length during release. If, however, the stiffness is measured by means of low frequencies quite a different stiffness-tension diagram is found. When the tension during stretch of the contracted fibres exceeds a certain magnitude, yielding is caused by the longitudinal vibrations, so that the stiffness is measured to be lower than it actually is, and lower during contraction than during rest. At release, however, the yielding has occurred, and the low frequencies cannot produce additional yielding, the course of the curve will be linear and the stiffness higher than at the same tension during extension. During these experiments the contraction is of course assumed to be continuous during extension as well as during release; the elastic locking of the contracted fibres is, as mentioned, irreversible only as long as the contraction lasts.

During contraction the stiffness thus exhibits a complicated dependence on the frequency on account of yielding, but when yielding is excluded the stiffness-tension diagram is found to be linear and the stiffness during contraction higher than at rest when referred to the same tension — this applying to skeletal muscles of the frog; the tension-stiffness diagrams for the cardiac muscles of the frog are on the other hand identical, whether the fibres are contracted or at rest (*Lundin*, 1944, pp. 52—53).

During contraction the stiffness of skeletal muscles is perceptibly dependent on temperature (*Buchthal & Kaiser*, 1944), the stiffness during contraction being higher at low temperatures than at high temperatures, which in turn means that the difference between stiffness at rest and during contraction measured at the same tension decreases with increasing temperature.

Mammalian muscle fibres exhibit completely identical values for stiffness during rest and contraction at the same tension — as appears from figs. 56—59 — and this applies to stiffness at a measuring frequency of 10 cycles per sec. as well as of 100 cycles per sec., the experiments being all carried out at a temperature of 37—38°C. The stiffness of contracted mammalian muscle fibres also increases linearly with the

tension when the frequency of the vibrations used for the measurements of the stiffness is 100 cycles per sec., so that the actual vibrations do not produce yielding. The curve in fig. 61 represents the mean values of six experiments on a fibre bundle which, during contraction, has been released and

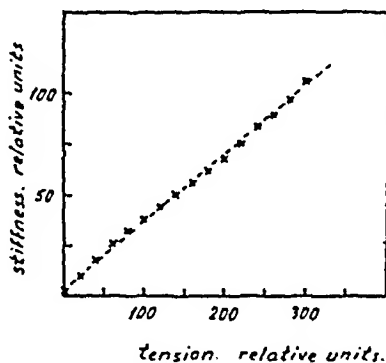


Fig. 61.

Stiffness of contracted fibres as a function of tension. Mean values from 6 single experiments on the same fibre bundle.

Preparation: fibre bundle from *m. gluteus max.* at length 60—130.

Abscissa: tension in relative units.

Ordinate: stiffness (determined at 100 cycles per sec.) in relative units.

stretched between length 126 and length 60. With regard to the viscous-elastic properties no fundamental difference thus exists between the skeletal muscle fibres of the guinea pig and of the frog.

The Modulus of Elasticity of the Muscle Fibres.

The modulus of elasticity calculated according to the static length-tension diagrams amounts for resting mammalian muscle fibres at equilibrium length on an average to 0.046×10^6 dynes \times cm.⁻² (mean of 14 experiments).

To check the results the static stiffness has been determined by means of two different experimental arrangements, and both experimental series have given practically the same result. The cross section of the fibre bundles has been calculated on the basis of the length and weight of the bundles. (The specific gravity of the muscular tissue is measured to be 1.06). In order also to check the determination of the cross section, the cross section has in five cases been determined both by calculation on the basis of length and

weight of the fibre bundle and by direct measurements of the diameters of the fibre bundle (d_1 and d_2) in two planes at right angles to each other, the cross section being calculated on the basis of the formula: $\pi \frac{d_1 \times d_2}{4}$. According to the first method the mean value of the cross section in these five cases is found to be 0.88 sq. mm. and according to the latter 0.7 sq. mm. Consequently this point does not seem to involve any serious source of error.

It is not possible to determine the static stiffness of contracted mammalian muscle fibres as the curve of the isometric maxima is not reversible. On the basis of the determination of the stiffness the modulus of elasticity may be calculated for the dynamic experiments (frequency 10 cycles per sec.) according to the formula: $\frac{\text{stiffness} \times \text{length}}{\text{cross section}}$, and is then found to be $0.052 \times 10^6 \text{ dynes} \times \text{cm.}^{-2}$ (mean of three experiments).

Buchthal (1942) found the modulus of elasticity of muscle fibres of the frog to be $0.5 \times 10^6 \text{ dynes} \times \text{cm.}^{-2}$ when determined by static experiments and $(0.81 \pm 0.11) \times 10^6 \text{ dynes} \times \text{cm.}^{-2}$ when determined by dynamic experiments.

It is not possible to explain the difference between the moduli of elasticity of muscle fibres from frogs and mammals by the different temperatures in which the two kinds of fibres work; the viscous-elastic level of muscles from frogs and mammals must be different, even if the contractile substances of the cross striated muscles of the two classes of animals otherwise behave uniformly.

D. Static Length-Tension Diagrams of the A- and I-Substance.

As shown in chapter III the anisotropic substance is elongated relatively more than the isotropic substance when the muscle fibres are stretched — in other words — the static stiffness of A is smaller than that of I.

Fig. 62 shows the correlation of the static length-tension diagram of the muscle fibres and the length of A and I as a function of stretch. On the basis of these diagrams it is possible to find the relationship between length and tension for A and I, as the tensions at the different degrees of stretch

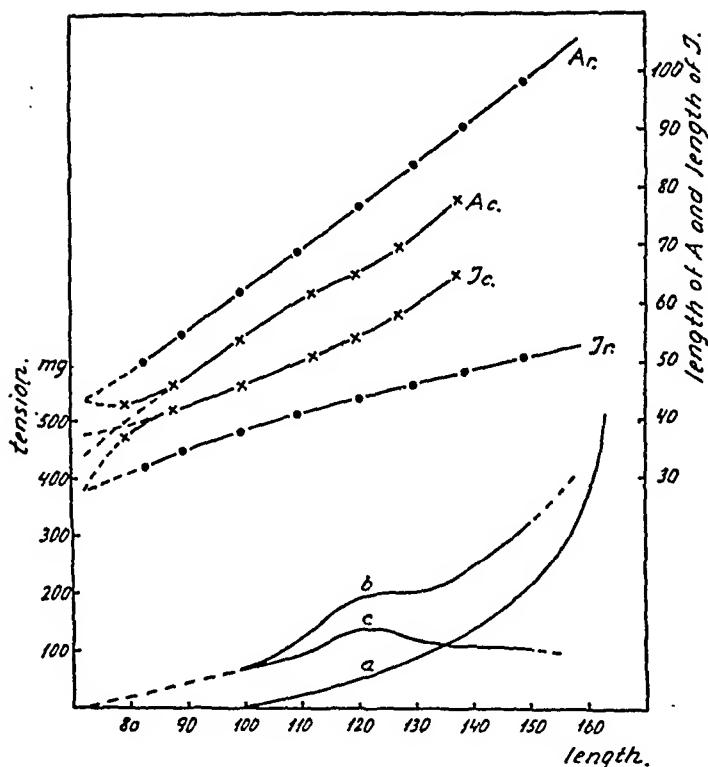


Fig. 62.

Relation of the length-tension diagram of the muscle fibres to the changes of the A- and I-substance during extension and contraction. See text p. 174.

The length-tension diagram: mean curve from fig. 43.

a) tension at rest. b) total tension (= tension at rest + extra-tension during contraction) for isometric tetanic contraction. c) extra-tension during contraction for isometric, tetanic contraction.

A- and I-measurements: mean values from all experiments on fibres from m. gluteus max.

A_r = A at rest (\cdot) I_r = I at rest (\cdot) A_c = A during contraction (\times) and I_c = I during contraction (\times).

Abscissa: length of fibre (equilibrium length = 100).

Ordinate: (to the left) length of A and I in per cent of the height of compartment at equilibrium length.

(to the right) tension in mg.

and the corresponding heights of the A- and I-substances are known. The heights of A and I are expressed in per cent of the total height of compartment at equilibrium length (2.24μ) in order to make the curves more comparable. The length-tension diagrams of A and I, plotted in this way, are shown in figs. 63 and 64, the curves show the resting values to have a uniform course, the static stiffness increases with increasing

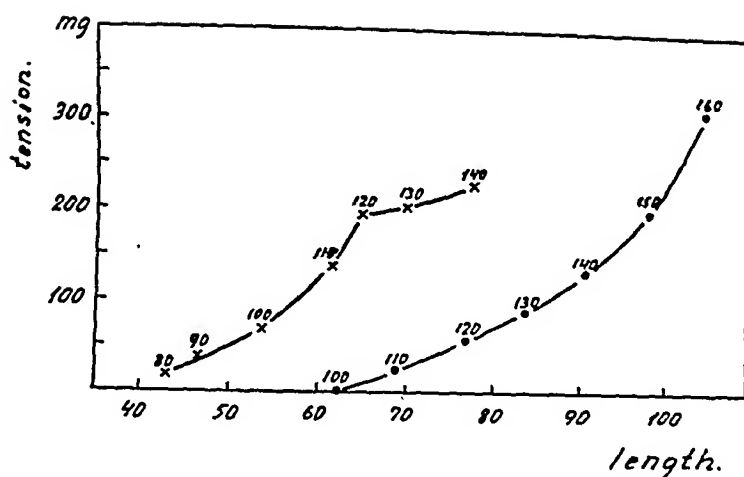


Fig. 63.

Length-tension diagram for the A-substance during rest and during contraction, see text p. 175. Curve of rest (•) and curve of the isometric maxima of the muscle fibres (×).

The figures denote the corresponding lengths of the muscle fibres (equilibrium length = 100).

Abcissa: length of A in per cent of the height of compartment at equilibrium length.

Ordinate: tension in mg.

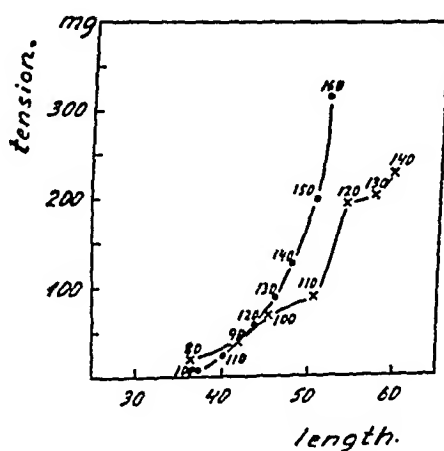


Fig. 64.

Length-tension diagram for the I-substance during rest and during contraction, see text p. 175. Curve of rest (•) and curve of the isometric maxima of the muscle fibres (×).

The figures denote the corresponding lengths of the muscle fibres (equilibrium length = 100).

Abcissa: length of A in per cent of the height of compartment at equilibrium length.

Ordinate: tension in mg.

stretch, the stiffness of A being — as mentioned — slightly smaller than that of I.

By histological experiments the percentage change of A and I respectively in case of isometric tetanic contraction is found to be identical, whether the contraction takes place at low or at high degrees of stretch (fig. 27). Apparently this does not conform with the investigations on the mechanical properties of the muscle fibres, which show that the extra-tension during contraction decreases at extensions above length 125. It is, however, not only measurements on mammalian muscle fibres which exhibit this apparent discrepancy. The same observation has been made by *Buchthal & Knappeis* (1943, b) during their experiments on fibres from frogs, and it will be discussed later on.

By means of contraction experiments performed on compressed fibres, it is found that during contraction I is always elongated in relation to its resting value at the same height of compartment, but that it may very well be shortened in relation to its height at equilibrium length. In these experiments it is, however, difficult clearly to account for the experimental conditions. The fibres are slightly compressed when contracting, and this compression may continue during the contraction. Adhesion to slides and similar conditions will be of more importance in these cases where the variations of the tension are so small; the actual tension of the individual fibre is consequently rather uncontrollable, and the experiments do not supply any proof of an active contraction of I, i. e. of a shortening of the equilibrium length of I during the contraction. In contrast with this the ratio A:I does not seem to change during contraction at the contraction equilibrium length, conf. the dotted curves of fig. 62. The other possibility, which has been indicated in the figure, is based on the assumption that during tensionless contraction the height of the I-substance is the same as the height of I at equilibrium length at rest. In that case the I-substance becomes higher than the A-substance during contraction. *Buchthal & Knappeis* found that this applies to muscle fibres of the frog in case of tensionless contraction (*Buchthal*, 1942, p. 51).

The length-tension diagrams of the A- and I-substance during contraction are also represented in figs. 63 and 64. They are calculated on the basis of the curves of fig. 62, the

height of A and I during contraction at the various degrees of stretch is stated in per-cent of the height of compartment at equilibrium length (2.24μ), and the corresponding contraction tension represents the mean curve of the "isometric maxima". The two substances behave differently during contraction.

The A-substance always assumes a shorter equilibrium length during contraction, and at equal lengths the tension of A is always higher during contraction than at rest. The difference in tension is greatest about the equilibrium length of the fibres. At length 120 of the fibres the gradient of the contraction curve of A decreases, which denotes a decreasing extra-tension during contraction.

With regard to I the tension at low degrees of stretch is higher during contraction than during rest, at high degrees of stretch it is lower during contraction than during rest — referred to the same heights of I at rest and during contraction. The point of intersection of the two curves is at a contraction value of I which corresponds to a contraction length of the fibres of 92 and at a resting length of 115. According to the diagram, contraction of mammalian muscle fibres which are released below equilibrium length (or more correctly below length 92) should thus cause an increase of the tension of I, which is higher than the increase of tension which would be produced by a corresponding passive extension. However, the curve is subject to an error, the contraction values of I being calculated on the basis of the curve of the isometric maxima, while I is actually stretched during contraction. The length-tension curve of contractions during which the height of the I-substance remains unchanged is not known, but a length-tension diagram of I plotted according to such a contraction curve would at any rate give lower values than the contraction length-tension curve shown in fig. 64. Consequently it has hardly been proved that I is able to exhibit a higher tension during contraction than during rest, when the fibres start contracting from a length below equilibrium length. There is, on the other hand, no doubt that, during contraction of fibres at equilibrium length or during contraction of stretched fibres, the I-substance is stretched more than it would be in case a corresponding passive tension was applied to it. This may be illustrated by an example: At equilibrium length the tension of I is zero at rest. Contraction at equilibrium length produces

(1943, a), and observed that a tension developed before any change in the cross striation was recorded, as it was impossible to record changes in cross striation from exactly the part of the fibre where the stimulation was applied. The contraction proceeds along the fibre at a rate of 0.32 m. per sec. (at 20°C.).

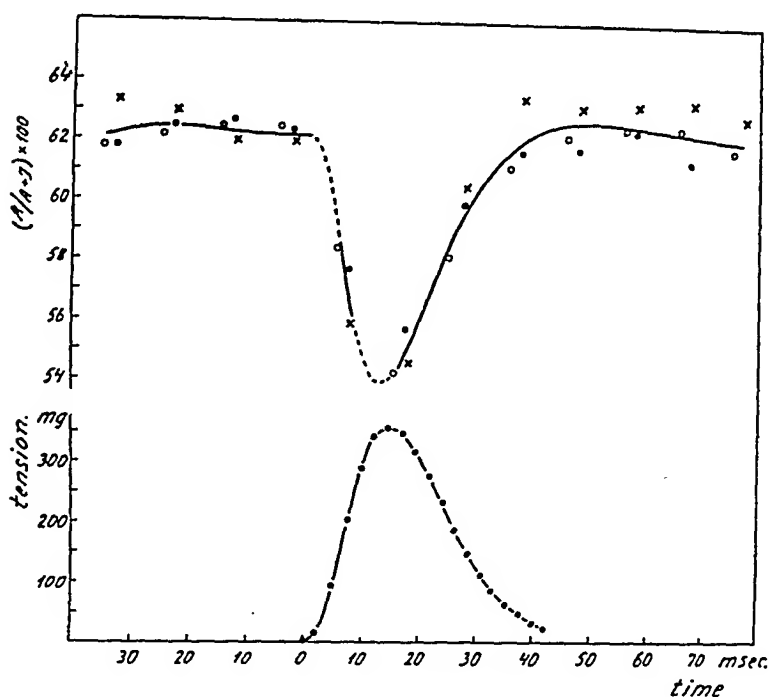


Fig. 65.

Relation of the variation in tension to the changes in the ratio $A : I$ during single contraction as a function of time.

Tension curve: single experiment on fibre bundle from *m. gluteus max.* at length 109.

$A : I$ curve: mean curve of 3 experiments on a single muscle fibre from *m. gluteus max.* at length 110.

Abscissa: time in msec.

Ordinate: $(A/A + I) \times 100$.

This fact is the reason why their later experiments (1943, b) showed that the peak tension does not occur until 20 msec. after the change in the cross striation has reached its maximum. The viscosity of the fibres is, however, also a contributory factor. Finally *Buchthal & Knappeis* observed that the tension persists for some time after the cessation of the optical changes. According to their opinion, the reason is that the stiffness increase in the I-substance during contraction subsides more slowly than the contraction changes of the

A-substance, so that I actually stretches A during the last phase of the contraction.

The experiments on mammalian muscle fibres have shown that the duration of the single contraction is the same, whether estimated on the basis of the variation of tension or on the basis of the alteration of cross striation. This is so far consistent with *Buchthal & Knappels'* hypothesis - - as it has already been shown that no increase of the stiffness of I occurs during contraction of the mammalian muscle fibres.

E. The Minute Structure.

On the basis of the various experiments on muscle fibres of the frog performed during the last decade at the Laboratory for the Theory of Gymnastics of the University of Copenhagen a mechanical equivalent of the minute structural unit of the muscle fibre has been composed - - a kind of model of a molecule or an aggregate of molecules; a combination of several of these units constitutes a micella. As a fundamental consistency has been found to exist between the muscle fibres of mammals and of frogs this mechanical equivalent should also apply to muscle fibres of mammals, and this chapter may be concluded with a description of this model and a discussion of the properties of the minute structure of the muscle.

The minute structural elements of the resting muscle fibres are longitudinally orientated; this can be seen i. a. from the investigations on birefringence and diffraction spectra and from the stiffness-tension diagrams. However, at the equilibrium length of the fibres the longitudinal orientation of the micellae, which consists of several chains of molecule aggregates, is not always complete, conf. the investigations on diffraction spectra and stiffness-tension diagrams. The chains in question are protein chains - - probably myosin, conf. the conformity between the birefringence of myosin and of muscular tissue. The minute structural unit is assumed to be a chain or spiral structure with an odd number of series of charges (at least three). When the fibre is at rest the spiral structure is kept stretched by the resultant attractive and repulsive central and angular forces between the series of charges (fig. 66) (conf. i a. *Buchthal & Lindhard*, 1939, pp 108 - 176).

To render the system stable when the chain is not completely extended there must be an odd number of series of charges.

In case of passive stretching of the fibres angular movements of the individual links of the spiral will occur — corresponding to the linear course of the stiffness-tension diagram. During contraction changes occur in the electric charge of the individual dipole, and the change is propagated along the chains. The attractive forces between the individual links are

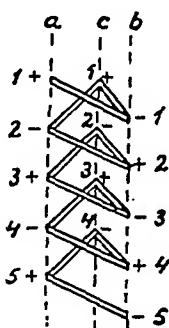


Fig. 66.

Model of the minute structure of muscle fibres, according to *Buchthal* (1942, p. 13).
 »Spiral« with three series of charges (a, b, and c).

increased and the "spiral" contracts, the equilibrium length decreases. The contraction ceases when the resting potentials have again been built up. When contraction occurs in a minute structural chain the process is always maximum — the individual chain following the "all or none" principle. If shortening of the chain is prevented during contraction, as e. g. in case of isometric contraction, tension will be developed and the resting parts of the chain will be stretched. The reason why the extra-tension during contraction decreases with increasing stretch is that the distance between the individual links of the "spiral" is increased by stretching, which has the effect that the contraction cannot proceed along the whole length of the chain, but only a small part of the latter will be able to participate in the contraction — resting substance is found in series with contracting substance. As shown in chapter III, fig. 27 such gradation cannot manifest itself by the optical changes in the ratio $A:I$ during contraction. By direct stimulation of the muscle fibres it is also possible to produce a gradation of the tension by varying the strength of the stimuli, and this gradation is due to the fact that the num-

ber of active minute structural chains may vary, in which case the resting substance "shunts" the contracted substance. This gradation is not within the limits of the optically recognizable either. It is not until the two types of gradation are combined that a recognizable variation of the A:I change during contraction is obtained. These facts were ascertained by *Buchthal & Knappeis* in their paper of 1943, a. Single contractions of fibres of the frog which are considerably stretched show a recognizably smaller change in the ratio A:I, when threshold stimuli are used than when maximum stimuli are used.

By stretching an already contracted fibre, it is possible to obtain a higher tension than in case of isometric contraction, the attractive forces between the individual links of the minute structural chains being larger during contraction when the electrical charges are closer to each other than in the resting position. When the tension during stretch of contracted fibres exceeds a certain magnitude some of the contracted links are torn from each other, thereby passing from a condition of contraction to a condition of rest — a yielding sets in, increasing the contraction equilibrium length of the fibre. The contracted fibre is locked in its equilibrium length as the transition links between contracted and resting elements of the minute structural chain will not attract each other until the contraction is discontinued. Yielding serves a purpose, in so far as it will cause the muscle fibres to exhibit fairly uniform tension at different degrees of stretch, and it will also serve as a kind of buffer when the muscle is suddenly contracted while subjected to a high load.

This model of the minute structure only applies to the anisotropic substance where the orientation of the structural elements is parallel, parallel orientation is an absolute condition of contractility. The I-substance may very well be of the same molecular structure as the A-substance, only the longitudinal orientation of the minute structure is not nearly as pronounced in the I-substance as in the A-substance (conf. *Hürthle's* model, 1931, b). In this way it is possible to explain the fibrillar structure of the muscle fibres and the polarization-optical difference between the A- and the I-substance.

Before dealing with the viscous-elastic properties of the minute structural elements of the muscle fibres, it should be emphasized that the muscle fibres are not homogeneous bodies, but that they consist of sarcolemma, myofibrils and sarco-

plasm, of alternate anisotropic and isotropic substances etc. The properties we are able to measure by means of stiffness determinations, damping experiments, etc. will consequently often be the resultants of the properties of different structural elements, a fact which complicates the investigation of the very structural element which it is our main purpose to determine: the contractile substance, even if the latter dominates the muscle fibre. Vice versa one substance may cause various of the properties of the muscle fibre, e. g. viscosity and elasticity; and these properties may merge into each other so smoothly that it is impossible to distinguish between them. We have, however, a series of experimental results which elucidates this problem, and a summary of these investigations will therefore be given.

As several times emphasized, the stiffness of the muscle fibres is a compound function of the elastic as well as of the viscous properties.

The purely elastic stiffness increases with the tension. The more the minute structural elements are straightened out by extension the higher their stiffness. During contraction the dynamic stiffness of fibres from frogs is increased. This increase can, to a great extent, be explained by the external increase of the tension produced by the contraction. The contraction, however, also produces an internal tension which we cannot determine, as we do not know the relationship between the active and the resting minute structural chains which "shunt" each other. Thus the internal tension causes an increase of stiffness which cannot be measured, a phenomenon which manifests itself in the observations made by *Buchthal & Kaiser*, (1944) that, in case of single contractions of fibres of the frog, the stiffness maximum will occur before the tension maximum, and that a particularly pronounced increase of stiffness will set in at the transition from rest to contraction. The actual contracted condition of the active minute structural elements will cause the stiffness to increase, the distance between the individual links of the spiral being diminished during contractions so that more powerful forces are set up between them.

The viscous properties of the muscle fibres are elucidated not only by stiffness determinations, but also by damping experiments and by investigating the elastic after-effects. The viscous properties of the resting muscle fibre are probably essen-

tially due to the changes in linkage, emphasized by *H. H. Weber* (1934—1941) and *Hill* (1939), changes which may be produced in the minute structure of the contractile substance by stretch and by tension. Transition from one linkage modification to another lasts some time and results in a small variation in the length of the minute structural elements. In this way a change in the linkages will manifest itself as an elastic after-effect, and, as further described by *Buchthal, Kaiser & Knappeis* (1944), it supplies a better explanation of the viscoelastic properties of the muscle fibres than an equivalent, as e. g. that of *Levin & Wyman* (1927) (fig. 53). The distinction between viscosity and stiffness is thus somewhat factitious, the two terms merely expressing slightly varying properties of the same structure.

When the fibres are stretched the viscosity increases (*Buchthal*, 1942, p. 100), the more the elements of the minute structure are stretched the more difficult it is to impose linkage variations on them. A transport of liquid will further take place between the A- and the I-substance in the resting muscle in case of alteration of length; we have seen that the relative height of the A- and the I-substance is altered — if only slightly — when the fibres are stretched, but no corresponding difference in the fibre diameters at A and I is observed. *Gasser & Hill* (1924) assumed this transport of liquid to be of great importance to the viscosity of the muscle fibres.

The viscous properties increase during contraction. This may be due to the above-mentioned transport of liquid between A and I, the relative volumes of both being altered during the process. *Buchthal* (1942, p. 106), however, is of the opinion that the transport can only be of minor importance. The increase of the viscous properties may also be due to the previously mentioned condition that not all of the minute structural chains are necessarily contracted, in which case the active chains will be "shunted" by the remaining resting chains. An actual internal friction will be set up between them. If whole muscles or larger fibre bundles are examined the possibility of an actual internal friction will be greater, as a displacement of fibres in relation to each other and to the connective tissue etc. will take place. It is finally probable that the contracted links of the chain are less liable to exhibit variations in the linkages than the resting parts and that, for this reason, the viscosity increases during contraction. In that case it also

becomes explicable that the viscosity of the contracted fibres decreases with stretching, a smaller part of the chains of the minute structure in the stretched fibres being in a contracted state on account of yielding.

While the viscous and elastic properties of resting muscle fibres of the frog are independent of temperature variations, the viscosity and the elastic stiffness of contracted fibres will show pronounced dependence on temperature (*Buchthal, Kaiser & Knappeis*, 1944), and this observation is correlated with the "propagated chain reaction" during contraction by *Buchthal, Kaiser & Knappeis*.

Mammalian muscle fibres exhibit the same dynamic stiffness at rest and during contraction, whether the stiffness is determined at a frequency of 10 or 100 cycles per sec. This may be explained by the temperature dependence of the contraction stiffness.

Buchthal was able to show that at low degrees of stretch the static stiffness of A, as well as that of the I-substance, increases during contraction; at higher degrees of stretch it is only the stiffness of the A-substance which increases. In mammalian muscles the I-substance does not, on the other hand, seem to contribute actively to the contraction. This may perhaps have some connection with the fact that the dynamic stiffness at rest and during contraction of the mammalian muscle fibres is uniform. It would, however, be desirable if this problem could be further elucidated by release-contraction experiments on mammalian muscle fibres. An investigation of the properties of mammalian muscles at low temperatures would also offer a possibility of extending the comparison between the mechanical properties of muscle fibres of the frog and of mammals, as it would then be possible to examine the muscle fibres of the two animal classes at the same temperature.

F. Conclusion.

Mammalian muscle fibres are high-elastic.

When stretched the resting muscle fibres only develop low tension in the beginning, at extension above length 150 the tension increases more rapidly — the static stiffness is increased.

If the fibres are stretched more than 40—50 per cent a plastic elongation of the fibres will set in, the equilibrium length is increased, but rarely more than 10 per cent.

Muscle fibres may be stretched up to 100 per cent without the structure or function being destroyed.

In case of isometric tetanic contraction the extra-tension during contraction is maximum at length 100—120, decreases with increasing extension and becomes zero at length 160—200.

When isometrically contracted muscle fibres are released the contraction equilibrium length amounts to about 72.

The increase of tension during single contraction amounts to one fourth to one third of the increase of tension during tetanic contraction.

In case of single contraction the increase of tension during contraction is also maximum at length 115—125.

The duration of a single contraction amounts to 35—45 msec. The peak tension is reached in the course of 15 msec.

With increasing degree of stretch the duration of the single contraction is increased, both with regard to the actual contraction phase and the relaxation phase.

Fatigue of the fibres will, on the other hand, result in decrease of the extra-tension during contraction, but will not result in a longer duration of the actual contraction phase.

If the muscle fibres are stimulated by single stimuli repeated with increasing frequency, a remaining tension will begin to develop at a frequency of 10 impulses per sec. The extra-tension of the single contraction decreases at a frequency of 16 and particularly at a frequency of 40. The tetanic contraction is complete at a frequency of 80—90.

The individual muscle fibre can produce an increase of tension of 8—10 mg. at isometric tetanic contraction.

Resting mammalian muscle fibres exhibit viscous resistance against alteration of the length, when the alteration takes place at a certain speed.

When contracted muscle fibres are stretched a plastic yielding will occur, increasing the contraction equilibrium length.

Release of contracted mammalian muscle fibres shows that the latter are elastically locked as long as the contraction lasts, as the equilibrium length during contraction does not decrease in spite of the release.

The tension developed by the fibres in case of isometric

contraction is smaller than the tension obtained by stretching the fibres during contraction to the same length, but higher than the tension developed by the fibres, if they are released from isometric contraction at a higher degree of stretch.

The dynamic stiffness of the muscle fibres ($\Delta \text{tension} / \Delta \text{length}$) increases linearly with the tension, which shows that the minute structural elements of the muscle fibres are completely longitudinally orientated; the variations in length are assumed to occur as angular movements of the minute structural elements.

Mammalian muscle fibres exhibit the same stiffness whether at rest or during contraction, when referred to the same tension.

By correlating the length-tension diagram to the changes which are found to occur in the ratio A:I during extension and contraction, it is shown that, at the same length, A yields a larger tension during contraction than during rest. With regard to I the opposite is the case, at any rate when the fibres are contracted at length 100 or more. The I-substance compromises the contraction either on account of reduced stiffness or on account of plastic elongation.

The modulus of elasticity of the mammalian muscle fibres is found to be $0.046 \times 10^6 \text{ dynes} \times \text{cm.}^{-2}$ by static experiments and $0.052 \times 10^6 \text{ dynes} \times \text{cm.}^{-2}$ by dynamic experiments.

The modulus of elasticity of the mammalian fibres is lower than that of fibres of the frog, but there is otherwise no fundamental difference between the mechanical properties of the two kinds of muscles, when allowing for the different temperatures at which the fibres work.

The mechanical equivalent of the minute structural unit of the muscle fibres of the frog also applies to the mammalian muscle fibres.

The viscous properties of the muscle fibres are assumed chiefly to be due to variations in linkage of the minute structural elements.

SUMMARY IN ENGLISH.

The views held by various investigators on the structure of the cross striated muscle fibres differ widely; one of the reasons for this is probably the prevailing use of fixed material for previous histological investigations. It is emphasized that the experimental conditions are more clearly defined when living, isolated muscle fibres are used; this does not only apply to analyses of the microscopical and submicroscopical structure of the muscle tissue, but also to investigations on the function of the muscle tissue. At the Laboratory for the Theory of Gymnastics, University of Copenhagen, such investigations on living, isolated, cross striated muscle fibres have been carried out on fibres of the frog, and the purpose of the present investigation is to study the structure and function of living, isolated, cross striated mammalian muscle fibres by corresponding methods, in order, i. a. to be able to compare conditions within the two animal classes.

In order to define the different structural elements a short summary of the structure of muscle tissue is given in *chapter I, section A*.

In *section B* the more significant previous investigations on the macroscopic and microscopic structure of muscle tissue are dealt with. A section is devoted to each of the individual structural elements, and it is endeavoured to correlate previous investigations with the author's own observations.

In the living, mammalian muscle fibres longitudinal, structural elements, the myofibrils, may be observed extending continuously throughout the length of the muscle fibre, but here it is impossible to ascertain any well-defined diameter of the myofibrils or arrangement of the myofibrils in uniformly dimensioned bundles. Myofibrils of a spiral course have not been observed either.

The A- and I-cross striae are due to periodical variations of the myofibrillar minute structure and a uniform arrangement of the myofibrils. As shown by W. J. Schmidt (1934) the isotropic segments also possess a slight anisotropy.

The investigations confirm that the Z-membranes are true structural elements; they appear more clearly when the fibres are stretched, but no proportionality is found between their thickness and the degree of stretch of the fibres. The exact structure of the Z-membranes is not fully elucidated, they seem to be an intrafibrillar as well as an interfibrillar structure.

Q_H , a lighter band in the central zone of the anisotropic cross striae, is often observed in living muscle fibres, most clearly in stretched fibres, it probably represents a real variation in the structure of the myofibrils. M, a fine, dark stripe in A, has also been observed in living, mammalian muscles, but the most frequent observation is narrow, dark cross striae, asymmetrically arranged in A, which are probably merely optical artefacts. N-cross striae, narrow, dark bands on both sides of Z have, however, not been observed in living, mammalian muscle fibres.

It should be emphasized that the cross striation of the muscle fibres according to *Buchthal & Knappeis'* investigations (1940) is not helicoidally arranged. "Nonius" periods and even sphenoids are observed in living muscle fibres, but this may be due to displacement of fibrils.

The various mammalian muscle fibres examined have displayed no differences, neither with regard to colour, transparency or mechanical properties (red-white or "trübe-helle" muscle fibres).

The sarcolemma, the connective tissue of the muscle, and the connection between myofibrils and tendons is discussed. It is considered most probable that the tension is transmitted from the contractile substance via the Z-membranes to the connective tissue of the muscle, even if certain observations do not agree with this theory. It is pointed out that the structure of the connective tissue allows for considerable displacements between the individual muscle fibres, but the anisotropic layers of neighbouring fibres are usually found to be opposite each other.

The motor end plates are only seen as blurred parts of living muscle fibres. Local application of a minute amount of

acetylcholine or dissolved potassium (abt. 10^{-5} mg. and 10^{-4} mg. respectively) will here act as a contraction-producing stimulus, exactly as in the experiments on lizards performed by *Buchthal & Lindhard* (1942).

Section C is a historical review of previous quantitative investigations on the ratio A:I and the changes in the latter during contraction.

Section D deals with previous investigations on the sub-microscopical structure of the muscle fibres, illustrated by optical, polarization-optical and roentgen-spectroscopical experiments and by examinations of the mechanical and thermo-elastic properties of the muscle fibres. These investigations all indicate that the contractile substance of the muscles is closely related to the myosin protein group. The birefringence of the muscle fibres consists of form birefringence and crystalline birefringence. In the isotropic segments of the myofibrils the micellae are not uniformly orientated, while this is the case in the anisotropic segments. The roentgen-spectroscopical and thermo-elastic properties of the muscle fibre indicate that the chain molecules of the contractile substance are flexible; while contraction causes a further folding of the chains they are straightened out when the muscle fibres are stretched. Investigations performed during the latest years on the correlation of the chemistry and physics of the muscle fibres are mentioned shortly.

Section E, the last section of chapter I, contains a summary of the changes which may be produced by chemical and physical agents in the muscle tissue, and it is shown that chemical fixatives may produce fairly pronounced changes in the structure, and that it is impossible to fix the muscle in a normal, contracted state. It is equally impossible to avoid alterations of the structure by using special fixing methods, as e. g. freezing, as recommended by *Hürthle*. Also within pathologico-anatomical research fixing may give rise to mistakes, as many of the so-called muscle degenerations are indistinguishable from artefacts. These observations could be confirmed by own experiments.

In *chapter II*, A the different methods and procedures used are described. After excision the muscle or the muscle fibres are placed in isotonic gum arabic-Ringer solution at a temperature of 37—38°C., the p_H of the solution is kept constant by a steady flow of oxygen and carbon dioxide. A fibre bundle

is isolated from the muscle, the bundle is then placed in a muscle chamber in which the final isolation down to fibre bundles consisting of one layer, or to individual fibres, takes place. In the chamber the preparation is kept fastened by metal clamps in such a way that it may be stretched or released. The electric stimulation is transmitted through the clamps. The muscle fibres are microphotographed and the height of the muscle compartment and the A- and I-layers measured on the negative films by means of an eyepiece screw micrometer. When the muscle fibres are examined in situ the abdominal wall of the test animal (guinea pig) is folded back on an especially designed microscope stage, and thin fibre bundles isolated in the muscle. The microscopical changes in the muscle fibres during single contractions are recorded as a function of time by means of *Buchthal & Knappeis'* technique (1943, a and b) — high pressure mercury lamp with high luminous intensity, rotating disc with apertures for admitting the light, and fall camera. With this apparatus a frequency of exposures of 100 per sec. and a time of exposure of less than 4 msec. are obtained.

The quantitative birefringence of the muscle fibres is determined according to the formula: $(n_a - n_o) = \frac{\gamma \lambda}{d}$, where $(n_a - n_o)$ is the birefringence, d the thickness of the object (in μ), γ the phase difference between the two refracted light rays, λ the wave length of the light used (in $m\mu$); the light employed is the green mercury line with $\lambda = 546 m\mu$. The phase difference ($\gamma\lambda$) is determined by means of Babinet's quartz wedge compensator. The fibre diameter is measured directly by means of an eyepiece screw micrometer.

For the investigation of the mechanical properties of the muscle fibres the author has used *Buchthal's* (1942, pp. 6—9) and *Buchthal & Kaiser's* (1944) set up. The latter permits simultaneous and continuous recording of length, tension and stiffness of the muscle fibres. Tension and variation of tension are recorded by means of the condenser myograph; longitudinal variations of the muscle fibres are produced by means of an oscillator and an electro-magnetic system.

Chapter II, B deals with examinations of the muscles of various animals (cat, rabbit, guinea pig, white mouse and rat), the object of which is to find the most suitable material for isolation of single muscle fibres. In order to be suitable for

this purpose the muscle must have a tendinous origin and insertion, its structure must not be too complicated, the amount of connective tissue must not be too abundant, the length of the fibres should be convenient and the muscle must be easily accessible. Among the muscles of the guinea pig the m. gluteus max., the m. serratus post. and the m. obliquus abd. intern. supply particularly convenient experimental material. The muscle fibres of mice and rats are on the whole too short. The structure of the muscles used is examined by maceration of the connective tissue through boiling. The thickness of the fibres varies considerably within each muscle, while the fibre diameters of the guinea pig muscles and of the muscles of the other mammals examined (cat, rabbit and goat) do not differ essentially. Compared with the muscles of the frog the muscle fibres of the guinea pig seem on the whole to be somewhat thinner and more uniform as to diameter.

In *chapter III* the results of the quantitative determinations of the height of muscle compartment and of A- and I-layers are given. The results of the examinations of the various guinea pig muscles used are all consistent, just as no differences are found between the muscle fibres from guinea pig, cat, rabbit and goat. The findings are the same whether excised fibres or fibres in situ are measured. At equilibrium length the height of compartment is $2.2\ \mu$, A amounts to 62—63 per cent of the height of compartment and I to 37—38 per cent. When the fibres are stretched the height of A increases relatively more than that of I, in case of 50 per cent extension A thus amounts to 65—66 per cent of the height of compartment. Examination of the fibres between crossed nicols gives the same results with regard to the ratio A : I. In case of isometric, tetanic contraction A is shortened by 14—15 per cent on an average, while the height of I increases by 21—25 per cent. The percentage changes in A and I during contraction are independent of the degree of stretch of the fibres. During single contraction the change in the ratio A : I lasts 30—40 msec., and the changes at the peak of the single contraction are just as pronounced as in case of tetanic contraction. The experiments yield the same results as the investigations performed with the same technique by *Buchthal, Knappeis & Lindhard* on muscle fibres of frogs and lizards. This indicates that the properties of the muscles of the different vertebrates are consistent. The highly differing observations made by

other authors are assumed to be due to the use of fixed muscles and, in case living muscle fibres have been used, to erroneous interpretation of the optical images and to the circumstance that artificial alterations in the structure have been considered to represent normal histological pictures.

As mentioned in *chapter IV* the birefringence of isolated living muscle fibres of guinea pigs is measured to be $(1.67 \pm 0.06) \times 10^{-3}$ and to be the same for fibres from m. gluteus max. and m. serratus post., slightly lower for thick than for thin fibres. The measurements performed by the author indicate that the birefringence increases when the fibre is stretched and that, with the method used, the fibres tend to become slightly flattened when stretched. The birefringence of the muscle fibres of guinea pigs is of the same magnitude as that of fibres of the frog, the birefringence of frog muscles being measured to be $(1.70 \pm 0.016) \times 10^{-3}$ by *Buchthal & Knappeis* (1938), who used the same technique. Later investigations by *Buchthal, Deutsch & Knappeis* (1946) with an improved technique have, however, shown that the birefringence, determined by a technique as that used by *Buchthal & Knappeis* (1938) and by the author of the present work, is 18.5 per cent too low, the exact birefringence of muscle fibres of the frog being measured to be 2.01×10^{-3} . Corrected according to these results the exact birefringence of mammalian muscle fibres is 1.98×10^{-3} .

Chapter V: The mechanical properties of the muscle fibres are investigated by means of static, semi-dynamic and dynamic experiments.

Section A deals with the static experiments by means of which the relationship between length and tension of the fibres is determined after cessation of the elastic after-effects. These experiments show that the fibres are highly elastic and that the static stiffness (Δ tension/ Δ length) increases with increasing stretch of the resting fibres. If the fibres are stretched more than 50 per cent a plastic elongation of the fibres will occur, the equilibrium length (i. e. the length at which the tension of the fibres is nil, but at which even the smallest elongation of the fibres will produce tension) being increased — usually not more than 10 per cent, however. The muscle fibres can be extended up to 100 per cent without deterioration of their structure or function.

The extra-tension during isometric tetanic contraction is

3—4 times higher than the extra-tension during isometric single contraction, and it holds for both types of contraction that the extra-tension during contraction is highest at length 100—125 (equilibrium length = 100). The individual mammalian muscle fibre can yield an extra-tension during tetanic contraction of 8—10 mg. The extra-tension during contraction decreases with increasing extension of the fibres and becomes nil at length 160—200. The contraction equilibrium length determined by release of the muscle fibres from isometric contraction is on an average 72, i. e. the fibres are shortened 28 per cent.

The duration of the single contraction of a mammalian muscle fibre amounts to 35—45 msec. (at 37°C.), the peak tension is reached in the course of 15 msec. With increasing degree of stretch of the fibre the actual contraction phase as well as the relaxation phase is increased, while fatigue results in a decrease of the extra-tension during contraction, not in a prolonged contraction phase.

When the muscle fibres are stimulated by single stimuli of increasing frequency a remaining tension will begin to develop at a frequency of 10 impulses per sec. The extra-tension of the single contraction decreases at frequency 16, but especially at frequency 40; at frequency 80—90 the tetanic contraction is complete.

Section B: It appears from semi-dynamic experiments in which the relationship between length and tension is recorded during the actual extension or relaxation of the fibres — the stretch and relaxation taking place at reduced speed — that resting muscle fibres exhibit a viscous resistance against alterations in length. When contracted muscle fibres are stretched a plastic yielding takes place so that the contraction equilibrium length is increased. Release of contracted mammalian muscle fibres show that they are elastically locked as long as the contraction lasts, as the contraction equilibrium length of the fibres do not decrease in spite of the release. The tension developed by the fibres during isometric contraction is lower than the tension obtained by stretching the fibres during contraction to the same length, but higher than the tension exhibited by the fibres if they are released from isometric contraction at a higher degree of stretch.

As mentioned in *section C* the dynamic stiffness of the muscle fibres increases linearly with the tension, which shows

that the minute structural elements are completely longitudinally orientated. The variations in length are due to angular movements in the minute structural elements. The dynamic stiffness of the mammalian muscle fibres is the same at rest and during contraction when referred to the same tension.

The modulus of elasticity of the mammalian muscle fibres has been measured to be 0.046×10^6 dynes \times cm.⁻² by static experiments and 0.052×10^6 dynes \times cm.⁻² by dynamic experiments.

Section D: By correlating the length-tension diagrams of mammalian muscle fibres with the measured changes in the ratio A:I during stretch and contraction it is found that at the same length A will exhibit greater tension during contraction than at rest. The opposite is the case with regard to I, at any rate when the fibres are contracted at or above the equilibrium length. The I-layer compromises the contraction either on account of reduced stiffness or on account of plastic elongation. In muscles of the frog an increase of the stiffness of I during contraction has, on the other hand, been observed in fibres extended up to length 150 (*Buchthal*, 1942, pp. 57—64).

In *section E* the results of the experiments on mammalian muscle fibres are compared with the previous investigations on muscle fibres of the frog, and the conclusion is drawn that, apart from the lower modulus of elasticity of the mammalian muscle fibres, there is no fundamental difference in the mechanical properties of the muscle fibres of the two animal classes, when allowing for the fact that the muscle fibres function at two different temperature levels.

A mechanical equivalent of the minute structural unit of the contractile substance of the muscle fibre is mentioned. The minute structural unit is assumed to be a spiral with an odd number or series of charges, the attractive and repulsive, central and angular forces of which keep the spiral stretched while at rest. Extension takes place in the shape of angular movements between the individual links of the spiral. In case of contraction the electrical charges of the individual dipoles change and the spiral contracts, the equilibrium length decreases. The viscous properties of the muscle fibres are assumed essentially to be due to variations in linkage of the minute structural elements of the contractile substance.

SUMMARY IN DANISH.

RESUMÉ.

De tværstribede Muskelfibres Struktur opfattes ret forskelligartet; dette maa bl. a. skyldes tidligere Undersøges overvejende Anvendelse af fixeret Materiale ved histologisk Undersøgelse. Det paapeges, at Forsøgsbetingelserne bliver mere veldefinerede, naar levende, isolerede Muskelfibre benyttes; det gælder ikke blot ved Analyse af Muskelvævets mikroskopiske og submikroskopiske Struktur, men ogsaa ved Undersøgelse af Muskelvævets Funktion. Paa Københavns Universitets gymnastik-teoretiske Laboratorium er saadanne Undersøgelser af levende, isolerede, tværstribede Muskelfibre foretaget med Frømuskler, og det er Formaålet med nærværende Arbejde paa tilsvarende Maade at undersøge Strukturen og Funktionen af levende, isolerede, tværstribede Pattedyrmuskelfibre for derved bl. a. at kunne sammenligne Forholdene hos de to Dyrerækker.

Til Definition af de enkelte Strukturelementer gives først i *Kapitel I Afsnit A* en kort Oversigt over Muskelvævets Struktur.

I *Afsnit B* gennemgaaes de vigtigste tidligere Undersøgelser over Muskelvævets makroskopiske og mikroskopiske Opbygning. De enkelte Strukturelementer diskuteres hver for sig, idet man samtidig søger at korrelere tidligere Undersøgelser med egne Iagttagelser.

I de levende Pattedyrmuskelfibre kan man se Longitudinalstrukturer, Myofibriller, der strækker sig kontinuerligt gennem Muskelfibrenes Længde; men derimod kan man ikke her erkende nogen veldefineret Myofibrildiameter eller Lejring af Myofibrillerne i ensartet dimensionerede Bundter. Man har heller ikke set spiralforløbende Myofibriller.

A- og I-Tværstribningen er knyttet til Myofibrillerne, skyldes periodisk Variation af den myofibrillære Finstruktur og

ensartet Lejring af Myofibrillerne. Som vist af W. J. Schmidt (1934) besidder de isotrope Segmenter ogsaa en ringe Grad af Anisotropi.

Undersøgelserne bekræfter, at Grundmembranerne (Z) er reelle Strukturer; de træder tydeligere frem ved Strækning af Fibrene, men man finder ikke Proportionalitet mellem deres Tykkelse og Fibrenes Strækningsgrad. Grundmembranernes nøjagtige Struktur er ikke fuldt klarlagt, det synes baade at dreje sig om intramyofibrillære og intermyofibrillære Dannelser.

QH, et lysere Baand i Midterzonen af de anisotrope Tværstriber, ses hyppigt i de levende Muskelfibre, tydeligst i strakte Fibre, det drejer sig sikkert om en reel Strukturvariation i Myofibrillerne. M, en smal og mørk Stribe i A, er ogsaa fundet i levende Pattedyrmuskler, men som oftest ses smalle Tværstriber lejret asymmetrisk i A, og det drejer sig da sikkert blot om optiske Artefakter. N-Tværstriber, smalle, mørke Baand paa hver sin Side af Z, har man derimod ikke set i de levende Pattedyrmuskelfibre.

Det maa fremhæves, at Muskelfibrenes Tværstrikning efter *Buchthal & Knappeis'* Undersøgelser (1940) ikke er helikoidal anordnet. Man kan se Noniusperioder og endda Sphenoder i de levende Muskelfibre, men dette kan fremkomme ved Fibrilfor-skydning.

De undersøgte Pattedyrmuskelfibre har ikke vist Forskel, hverken med Hensyn til Farve, Klarhed eller mekaniske Egenskaber (røde ~ hvide, respektive »trübe ~ helle« Muskelfibre).

Sarcolemma, Muskulens Bindevæv og Myofibril-Sene-Overgang diskuteres. Det anses for sandsynligst, at Spændingen overføres fra den kontraktile Substans via Grundmembranerne til Muskulens Bindevæv, selvom visse Fund er daarligt forenelige med denne Teori. Det paapeges, at Bindevævsstrukturen tillader stor Forskydelighed mellem Muskelfibrene indbyrdes, men man finder som oftest, at de anisotrope Lag ligger ud for hinanden i Nabofibre.

De motoriske Endeplader ses kun som Uklarheder paa de levende Muskelfibre, og lokal Applikation af en yderst ringe Mængde Acetylcholin eller Kalium i Opløsning (henholdsvis ca. 10^{-6} mg. og 10^{-4} mg.) virker her som kontraktionsfremkaldende Irritant, ganske som ved Firbenforsøgene foretaget af *Buchthal & Lindhard* (1942).

Afsnit C er en historisk Gennemgang af tidligere kvantita-

tive Undersøgelser over A/I-Forholdet og dettes Ændring under Kontraktion.

I *Afsnit D* omtales tidligere Undersøgelser over Muskel-fibrenes submikroskopiske Struktur, dels belyst ved optiske, polarisationsoptiske og røntgen-optiske Undersøgelser, dels belyst ved Undersøgelse af Muskelfibrenes mekaniske og thermoelastiske Egenskaber. Disse Undersøgelser tyder alle paa, at den kontraktile Substans i Musklerne er identisk med Myosin. Muskelfibrenes Dobbeltbrydning bestaar baade af Formdobbeltbrydning og Egendobbeltbrydning. I Myofibrillernes isotrope Segmenter er Micellerne ikke orienteret ensartet, medens dette er Tilfældet i de anisotrope Segmenter. Muskelfibrenes røntgen-optiske og thermoelastiske Egenskaber tyder paa, at Molekylkæderne i den kontraktile Substans er flexible; medens Kontraktion medfører en yderligere Sammenfoldning af Kæderne, udrettes de ved Strækning af Muskelfibrene. — De sidste Aars Undersøgelser over Korrelationen mellem Muskelkontraktionens Kemi og Fysik omtales kort..

Med *Afsnit E* afsluttes Kapitel I med en Gennemgang af de Forandringer, som kan fremkaldes ved kemisk og fysisk Paavirkning af Muskelvævet, og det vises, at kemiske Fixeringsmidler kan fremkalde ret udtalte Ændringer i Muskelstrukturen, og at det ikke er muligt at fixere Musklen i dens normale Kontraktionstilstand. Heller ikke specielle Fixeringsmetoder som f. Eks. den af *Hürthle* anbefalede Frysning giver uærdret Struktur. Ogsaa inden for den pathologisk-anatomiske Forskning kan Fixering give Anledning til Fejltagelser, idet mange af de saakaldte Muskeldegenerationer ikke kan skelnes fra Artefakter. Alle disse Forhold belyses ved egne Forsøg.

I *Kapitel II A* beskrives den udarbejdede Teknik. Efter Excisionen holdes Musklen resp. Muskelfibrene opbevaret i 37—38° opvarmet, isotonisk Gummi-Ringer- eller Polyviol-Ringer-Opløsning med kontrolleret pH, under konstant Ilt-Kuldioxid-Tilledning. Først præpareres et Fiberbuntt fri fra Musklen; dette anbringes i et Præparatkammer, hvor den endelige Præparation ned til det eenlagede Fiberbuntt eller den enkelte Muskelfiber finder Sted. I Præparatkammeret holdes Præparatet fæstet ved Hjælp af Metalklemmer, saaledes at det kan strækkes og afspændes. Den elektriske Irritation sker via Metalklemmerne. Muskelfibrene er mikrofotograferede, og paa Negativfilmen er Højden af Muskelfaget og af A- og I-Lagene

maalt ved Hjælp af et Okularskruemikrometer. Hvor Muskelfibrene er undersøgt in situ, er Forsøgsdyrets (Marsvinets) Abdominalvæg klappet ud over et særlig indrettet Mikroskopobjektbord, og tynde Fiberbundter er udpræpareret i Muskulaturen. Registrering af de tidsmæssige, mikroskopiske Forandringer i Muskelfibrene under Enkelkontraktion er sket ved Hjælp af *Buchthal & Knappeis'* Forsøgsteknik (1943, a og b) — Kviksølv-Højtrykslampe med stor Lysstyrke, roterende Lysblænde og Faldkamera. Hermed kan man opnaa en Billedhyppighed paa 100 Billeder pr. Sec. og en Exponeringstid paa mindre end 4 Millisec.

Muskelfibrenes kvantitative Dobbeltbrydning er beregnet efter Formlen: $\left(n_a - n_o = \frac{\gamma \lambda}{d} \right)$, hvor $n_a - n_o$ = Dobbeltbrydningen, d = Objektets Tykkelse (i μ), γ = Fasedifferensen mellem de to forskelligt brudte Straaler, λ = Bølgelængden af det anvendte Lys (i $m\mu$); der valgtes den grønne Kviksølvlinie svarende til $\lambda = 546 m\mu$. Gangforskellen ($\gamma\lambda$) er bestemt ved Hjælp af Babinet's Kvartskilekompensator, Fiberdiametere er maalt direkte ved Hjælp af Okularskruemikrometer.

Til Undersøgelse af Muskelfibrenes mekaniske Egenskaber er *Buchthal's* (1942, p. 6—9) og *Buchthal & Kaiser's* (1944) Forsøgsanordning benyttet. Denne tillader samtidig og kontinuerlig Registrering af Længde, Spænding og Stivhed af Muskelfibre. Ved Hjælp af Kondensatormyograf registreres Spænding og Spændingsvariation; ved Hjælp af Tongenerator og et elektromagnetisk System fremkaldes Længdesvingninger af Muskelfibre.

I *Kapitel II B* omtales hvilke Muskler i de undersøgte Forsøgsdyr (Kat, Kanin, Marsvin, hvid Mus og Rotte), der er fundet bedst egnet som Materiale til Isolering af enkelte Muskelfibre. For at være velegnet til dette Formaal skal Musklen helst have senet Udspring og senet Fæste, have en ukompliceret Opbygning med ringe Bindevævsindhold og passende Fiberlængde, samt være let tilgængelig for Excision. Af Marsvinets Muskler er især m. gluteus max., m. serratus post. og m. obliquus abd. intern. velegnet som Forsøgsmateriale. Muskelfibre i Mus og Rotter er gennemgaaende for korte. Opbygningen af de anvendte Muskler er undersøgt ved Maceration af Bindevævet ved Kogning. Inden for samme Muskel finder man ret stor Variation af Fibertykkelsen, derimod er der ikke væsentlig Forskel paa Størrelsen af Fiberdiametrene i de un-

dersøgte Marsvinemuskler og i Musklerne fra de andre undersøgte Pattedyr: Kat, Kanin og Ged. Sammenlignet med Frøens Muskler synes Marsvinets Muskelfibre gennemgaaende at være noget tyndere og mere ensartet i Diameter.

I *Kapitel III* omtales Resultaterne af de kvantitative Bestemmelser af Muskelfågets og A- og I-Lagenes Højde. Samtlige undersøgte Muskler fra Marsvin viser overensstemmende Forhold, ligesom der ikke er Forskel i Muskelfibre fra Marsvin, Kat, Kanin og Ged. Fundene er de samme, om der maales paa exciderede Fibre eller paa Fibre in situ. Ved Ligevægtslængde er Faghøjden $2,2 \mu$, A udgør 62—63 % af Faghøjden og I 37—38 %. Ved Strækning forlænges A relativt mere end I, ved 50 %'s Strækning af Fibrene udgør A saaledes 65—66 % af Faghøjden, I 34—35 %. Undersøgelse af Fibrene imellem krydsede Nicol'ske Prismer har givet samme Resultater med Hensyn til A/I-Forholdet. Ved isometrisk, tetanisk Kontraktion forkortes A gennemsnitligt 14—15 %, medens I tiltager 21—25 % i Højde. De procentiske Ændringer af A og I under Kontraktion er uafhængige af Fibrenes Strækningsgrad. Under Enkeltkontraktion varer Ændringen af A/I-Forholdet 30—40 Millisec., og Forandringerne er ved Enkeltkontraktionens Maximum ligesaa udtalte som ved tetanisk Kontraktion. Forsøgene har vist det samme som *Buchthal, Knappeis & Lindhard's* Undersøgelser af Frø- og Firbenmuskler (1936), foretaget med samme Metodik. Dette tyder paa, at der er Overensstemmelse mellem de forskellige Hvirveldyr's Muskler. Andre Forfatteres stærkt varierende Fund tilskrives Anvendelse af fixeret Materiale og, hvor der er benyttet levende Muskelfibre, Fejltydning af de optiske Billeder og Tolkning af artificielle Strukturforandringer som normale histologiske Billeder.

Som nævnt i *Kapitel IV* maales Dobbeltbrydningen af de isolerede, levende Muskelfibre fra Marsvin til $(1,67 \pm 0,06) \times 10^{-3}$, ens for Fibre fra m. gluteus max. og m. serratus post., lidt lavere for tykke end for tynde Fibre. Forfatterens Maalinger tyder paa, at Dobbeltbrydningen tiltager ved Strækning, og at Fibrene ved den benyttede Metodik afflades let ved Strækning. Dobbeltbrydningen af Muskelfibre for Marsvin er af samme Størrelsesorden som Dobbeltbrydningen af Frøfibre, der af *Buchthal & Knappeis* (1938) maalttes til $(1,70 \pm 0,016) \times 10^{-3}$ med samme Teknik. Senere Undersøgelser af *Buchthal, Deutsch & Knappeis* (1946) med forbedret Teknik har dog

vist, at Dobbeltbrydningen, bestemt ved den af *Buchthal & Knappeis* (1938) og den af Forf. anvendte Teknik, ligger 18,5 % for lavt, idet Frømuskelfibrenes nøjagtige Dobbeltbrydning maales til $2,01 \times 10^{-3}$. Korrigeret efter disse Tal, bliver Pattedyrmuskelfibrenes nøjagtige Dobbeltbrydning $1,98 \times 10^{-3}$.

Kapitel V: Muskelfibrenes mekaniske Egenskaber er undersøgt ved statiske, semidynamiske og dynamiske Forsøg.

I *Afsnit A* omtales de statiske Forsøg, hvor Forholdet mellem Fibrenes Længde og Spænding er bestemt, efter at de elastiske Eftervirkninger er ophørt. Disse Forsøg viser, at Fibrene er højelastiske, og at den statiske Stivhed (Δ Spænding/ Δ Længde) tiltager ved Strækning af de hvilende Fibre. Strækkes de over 50 %, indtræder der en plastisk Forlængelse af Fibrene, deres Ligevægtslængde (d. v. s. den Længde, hvor Fibrene har Spænding 0, men hvor de udvikler Spænding ved en selv nok saa lille Længdeforøgelse) forøges — dog sjældent mere end 10 %. Muskelfibrene taaler at strækkes op til 100 %, uden at deres Struktur eller Funktion ødelægges.

Kontraktionsextraspændingen ved isometrisk tetanisk Kontraktion er 3—4 Gange større end ved isometrisk Enkeltkontraktion, og for begge Kontraktionsformer gælder det, at Kontraktionsextraspændingen er størst ved Længde 100—125 (Ligevægtslængde = 100). Den enkelte Pattedyrmuskelfiber kan præstere en Extraspænding ved tetanisk Kontraktion paa 8—10 mg. Ved stigende Strækning af Fibrene aftager Kontraktionsextraspændingen for at blive 0 ved Længde 160—200. Kontraktionsligevægtslængden, bestemt ved Afspænding af Muskelfibrene fra isometrisk Kontraktion, er gennemsnitlig 72, d. v. s. Fibrene er forkortet 28 %.

Varigheden af Pattedyrmuskelfibrenes Enkeltkontraktion beløber sig til 35—45 Millisec. (ved 37°); Spændingsmaximum naas i Løbet af 15 Millisec. Ved stigende Strækningsgrad af Fibrene forlænges saavel den egentlige Kontraktionsfase som Afslapningsfasen, hvorimod Træthed medfører Aftagen af Kontraktionsextraspændingen, ikke længerevarende Kontraktionsfase.

Ved Irritation af Muskelfibrene med Enkeltirritamenter med stigende Frekvens stiger Muskelfibrenes Grundspænding ved en Frekvens paa 10 Impulser pr. Sec., Enkeltkontraktionens Extraspænding aftager ved Frekvens 16, men især ved

Frekvens 40; den tetaniske Kontraktion er fuldstændig ved Frekvens 80—90.

Afsnit B: Af semidynamiske Forsøg, hvor Forholdet mellem Længde og Spænding registreres under selve Strækningen eller Afspændingen af Muskelfibre, der foregaar med begrænset Hastighed, fremgaar, at hvilende Muskelfibre udviser en viscøs Modstand mod Længdeændring. Ved Strækning af kontraherede Muskelfibre indtræder der en plastisk Skridning, saaledes at Kontraktionslignevægtslængden forlænges. Afspænding af kontraherede Pattedyrmuskelfibre viser, at de er elastisk aflaaede, saa længe Kontraktionen vedvarer, idet Fibrenes Kontraktionslignevægtslængde ikke aftager trods Afspændingen. Den Spænding, Fibrene udvikler ved isometrisk Kontraktion, er mindre end den Spænding, man opnaar ved Strækning af Fibrene under Kontraktion til samme Længde, men større end den Spænding, Fibrene præsterer, hvis de er afspændt fra isometrisk Kontraktion ved en større Strækningsgrad.

Som omtalt i *Afsnit C* stiger Muskelfibrenes dynamiske Stivhed lineært med Spændingen, hvilket viser, at de finstrukturelle Elementer er fuldstændig længdeorienterede; Længdevariationerne foregaar som Vinkelbevægelser i de finstrukturelle Elementer. Pattedyrmuskelfibrenes dynamiske Stivhed er ens under Hvile og under Kontraktion henført til samme Spænding.

Pattedyrmuskelfibrenes Elasticitetsmodul er maalt til $0,046 \times 10^6$ dyn/cm.² ved statiske Forsøg, $0,052 \times 10^6$ dyn/cm.² ved dynamiske Forsøg.

Afsnit D: Ved Korrelation af Pattedyrmuskelfibrenes Længde-Spændingsdiagram med de maalte Ændringer i A/I-Forholdet under Strækning og under Kontraktion finder man, at A ved samme Længde præsterer større Spænding under Kontraktion end under Hvile. For I's Vedkommende er det modsatte Tilfældet, i hvert Fald ved Kontraktion af Fibre ved Lignevægtslængde eller derover. I kompromitterer Kontraktionen enten paa Grund af nedsat Stivhed eller paa Grund af plastisk Forlængelse. I Frømuskler er der derimod paavist en Stivhedsforøgelse af I under Kontraktion af Fibre ved Længde helt op til 150 (*Buchthal*, 1942, p. 57—64).

I *Afsnit E* sammenlignes Resultaterne fra Forsøgene med Pattedyrmuskelfibre med de tidligere Undersøgelser over Frømuskelfibre, og det konkluderes, at bortset fra Pattedyr-

muskelfibrenes lavere Elasticitetsmodul er der ikke principiel Forskel paa de mekaniske Egenskaber af Muskelfibre fra de to Dyrerækker, naar blot det tages i Betragtning, at Muskelfibre arbejder i forskelligt Temperaturniveau:

Et mekanisk Ækvivalent for den finstrukturelle Enhed i Muskelfibrenes kontraktile Substans omtales. Denne tænkes opbygget som en Spiral med et ulige Antal Ladningsrækker, hvis tiltrækkende og frastødende Central- og Vinkelkræfter holder Spiralen udspændt under Hviletilstand. Strækning foregaar som Vinkelbevægelser imellem de enkelte Led af Spiralen. Ved Kontraktion ændres de elektriske Ladninger i det enkelte Dipolelement, og Spiralen trækkes sammen, Ligevægtslængden aftager. Muskelfibrenes viscøse Egenskaber skyldes overvejende Bindingsvariationer i den kontraktile Substans' Finstruktur.

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THE HISTORY OF THE UNITED STATES

CHAPTER I

The first part of the history of the United States is the discovery of the continent by Christopher Columbus in 1492. This event marked the beginning of European settlement in North America. The second part of the history is the period of exploration and settlement by other European powers, including France, Spain, and the Netherlands. The third part of the history is the period of the American Revolution, which began in 1776 and ended in 1781 with the signing of the Treaty of Paris.

The fourth part of the history is the period of the early republic, which began in 1789 with the signing of the Constitution and ended in 1800 with the election of Thomas Jefferson as president. The fifth part of the history is the period of the Louisiana Purchase, which was completed in 1803. The sixth part of the history is the period of the War of 1812, which began in 1812 and ended in 1815 with the signing of the Treaty of Ghent.

The seventh part of the history is the period of the antebellum era, which began in 1815 and ended in 1861 with the outbreak of the Civil War. The eighth part of the history is the period of the Civil War, which began in 1861 and ended in 1865 with the signing of the Emancipation Proclamation. The ninth part of the history is the period of Reconstruction, which began in 1865 and ended in 1877 with the signing of the Reconstruction Act.

The tenth part of the history is the period of the Gilded Age, which began in 1877 and ended in 1900 with the signing of the Sherman Antitrust Act. The eleventh part of the history is the period of the Progressive Era, which began in 1900 and ended in 1914 with the signing of the Clayton Antitrust Act. The twelfth part of the history is the period of World War I, which began in 1914 and ended in 1918 with the signing of the Treaty of Versailles.

The thirteenth part of the history is the period of the Roaring Twenties, which began in 1918 and ended in 1929 with the signing of the Smoot-Hawley Tariff Act. The fourteenth part of the history is the period of the Great Depression, which began in 1929 and ended in 1933 with the signing of the New Deal. The fifteenth part of the history is the period of World War II, which began in 1939 and ended in 1945 with the signing of the Potsdam Declaration.

The sixteenth part of the history is the period of the Cold War, which began in 1945 and ended in 1991 with the signing of the Maastricht Treaty. The seventeenth part of the history is the period of the post-Cold War era, which began in 1991 and continues to the present. The eighteenth part of the history is the period of the 21st century, which began in 2001 and continues to the present.

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ULTRAVIOLET IRRADIATION
WITH
ARTIFICIAL ILLUMINATION

A TECHNICAL, PHYSIOLOGICAL, AND
HYGIENIC STUDY

BY

HANS E. RONGE

STOCKHOLM 1948

Errata.

Page 22, 7th line from below,

for E. P. WIDMARK, read E. J. WIDMARK

Page 29, 9th line from above,

for 1822, read 1882

Page 51, 8th line from below,

for 340—880 mμ, read 340—380 mμ

Page 86. Formula (6) should be

$$(6) \quad z = k (1 - e^{-k - a^{\tau}})$$

Page 108. The uppermost formula should be

$$\sigma^2 = \frac{(n_1 - 1) \sigma_1^2 + (n_2 - 1) \sigma_2^2}{n_1 + n_2 - 2}$$

Page 157. Formula (8) and (9) should be completed according to

$$(8) \quad \frac{P}{P_0} = e^{-\frac{F \cdot R}{V} \cdot t}$$

$$(9) \quad \frac{P}{P_0} = e^{-\frac{F \cdot R}{U} \cdot t}$$

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Uppsala, March 1948.

H. E. R.

I.

Introduction.

CHAPTER 1.

The Basis and Purpose of the Investigation.

During the last few decades there has been rapid development in the field of artificial lighting. An outstanding progress is the possibility to control to a greater extent the spectral composition of radiant energy for illumination. This advance was reached by the introduction of gaseous-discharge lamps and fluorescent lamps in lighting practice. The spectral control has hitherto been applied mainly within the limits of the visible spectrum, with the development of "daylight illuminants". Early in this new era of illumination the suggestion was made, and experimentally realized, to utilize the technical possibility existing for the accomplishment of artificial daylight also with respect to the invisible components of natural daylight, primarily the ultraviolet radiation (LUCKIESH, 1930, 1946; PORTER, EGELER, and STURROCK, 1932; ODAY and PORTER, 1933). The idea has, however, not yet been investigated with respect to possible hygienic and physiological value and practical applicability. This has been the main purpose of the investigation presented here.

The primary reason for the study is found in the fact that important photobiological effects of daylight and sunlight, such as sunburn and synthesis of vitamin D, are limited to the ultraviolet part of the sun's spectrum and especially the very short-wave end of it. Outdoor natural daylight involves, for human beings, not only an illumination effect but also an irradiation effect, and indoor artificial illumination will never be an ideal substitute for daylight unless both of these general effects are secured. The natural daylight inside a common window is almost completely deprived of these particular rays, due to a selective absorption in the glass. There is in this respect a kind of biological shadow from ordinary windows, and even internal daylight is not a complete substitute for outdoor daylight.

In the interpretation of the ultraviolet irradiation effect of daylight an important geographical factor must be considered. In most inhabited parts of the earth the problem of the people might rather be to protect themselves against this kind of radiation; at least, there is no need of any supplementary irradiation. For the Scandinavian peoples, on the other hand, the natural supply of ultraviolet radiation might be expected to be less than for any other nation. The reason is that the short-wave ultraviolet part of the solar radiation is depleted to a much greater extent than the visible and the infra-red parts by the low sun elevations predominant there. The latitudes of Scandinavia, 55—70 deg. N., correspond to those of South Greenland, Alaska and Siberia, and the life of the Scandinavian nations is primarily dependent on the indirect supply of heat from ocean currents and atmospheric convection. In this way the climate of the air makes settling possible (cf. MARKHAM, 1947), but the solar radiation climate may be expected to be less favourable, especially with respect to the supply of ultraviolet radiation during the long winter. The problems concerning the possible hygienic importance of a supplementary administration in some form of ultraviolet radiation may then be considered as a special, or specific, interest of Scandinavia.

There is, on the other hand, undoubtedly manifest a tendency to exaggerate the vital importance of both natural and artificial sunlight and, especially, to make extravagant claims for it as a therapeutic force (cf. LAURENS, 1935). Among the variety of effects hitherto recognized of ultraviolet energy on the human body only the photo-synthesis of vitamin D in the skin is proved as an essential. It is a generally accepted fact that the ultraviolet component of daylight constitutes the main natural source of this vitamin for both man and animals. The pronounced geographical and seasonal variation of the incidence of rickets in infants may receive a complete explanation on this basis. The main action of vitamin D in the human body is to promote the calcification of the bones, and the vitamin may then be assumed to be of special importance for the growing body. The potential risk of a vitamin D deficiency in infants during the long winter in Sweden is now well recognized and, in the main, is successfully counteracted by the administration of vitamin D preparations to all babies and infants. In older infants, as school children, the clinical manifestations of a possible vitamin D defi-

ciency are not very marked, and even rather unknown, and prophylactic measures have not the same prevalence. Hence, school children provide a suitable experimental material for an investigation on both the physiological consequences of the lack of natural ultraviolet radiation and the possible effects of a supplementary administration of it during the winter. The mechanisms underlying the radiation effects are secondary questions in this respect, and are not the subjects of the present investigation. The possibility may not *a priori* be excluded that a continuous ultraviolet irradiation of children also exerts other effects of health-improving character than does a simple administration of pure vitamin D. The present state of our knowledge — or rather our lack of exact knowledge — in this field does not justify a presumptuous dismissal of ultraviolet radiation as an additional factor of hygienic and biological importance.

In contrast to the visible and infrared components of sunlight, no immediate sensation exists for man when the ultraviolet component is lacking; for light and radiant heat the body is provided with special sense organs, but ultraviolet radiation acts in complete silence. This adds further importance to ultraviolet as a potential factor in the radiation climate, especially from the hygienic point of view. The most rational way of securing people their possible requirement of ultraviolet in the dark season should then be to combine it inherently with its perceptible relevants, i. e., to utilize the obvious claim for artificial illumination during the winter as a means for the regulation of the silent need of ultraviolet radiation. Indoor artificial lighting should provide a *radiation climate* with as much as possible of the biological effects of outdoor natural daylight. The special feature of such a manner of administration should be the continuous low-intensity irradiation, in the place of the massive high-intensity exposures used in physical medicine and in the common form of artificial sunlight treatments.

Besides the direct effects on the occupants it may be expected that an artificial lighting system with the simultaneous emission of ultraviolet radiation also exerts a favourable bactericidal action in the room. The effect in this respect of common artificial illumination is practically none owing to the low intensity and the lack of powerful ultraviolet radiation. Natural daylight from windows maintains some part of the germicidal power, due to the comparatively high intensity of blue and near-ultraviolet radiation (cf. Hol-

LAENDER, 1946). The most effective radiation for killing bacteria is, however, the ultraviolet wavelengths corresponding to the short-wave limit of the solar spectrum, and the maximum effectiveness is found at still shorter wavelengths. This germicidal power of short-wave ultraviolet radiation has in recent years been effectively utilized as a basis for sanitary control of air-borne infections, furthered especially by the development of the low-pressure mercury lamp (cf. MOULTON, 1942). An ultraviolet illumination as outlined here would combine the germicidal effect in the room with the irradiation effect on the occupants.

The International Commission on Illumination (I. C. I.) has constituted at the 1931 Cambridge congress a Committee on Ultraviolet Radiation, with the programme to evaluate physical measurements of ultraviolet radiation as emitted by modern light sources. The Reports of the Secretariat-Committee to the latest congresses, 1935 and 1939 (I. C. I., 1937, 1942), deal particularly with problems concerning the application and standardization of spectral measurement devices, but there are also collected data and discussion on the biological effects of ultraviolet radiation, which are of importance to the physical aspects of an 'ultraviolet illumination.' In the last report (I. C. I., 1942) it is emphasized that "It is for the medical science to express views as to the desirability of the general use of sources of ultraviolet radiation"; the present investigation may be considered as a first attempt to give an experimentally founded answer to this basic problem of the field.

CHAPTER 2.

A Brief Survey of Ultraviolet Radiology.

The field of ultraviolet radiology involves aspects from a number of different sciences, as physics, chemistry, technology, climatology, biology, hygiene, and medicine, and most of them are in some form represented also in the present study. It is, however, far beyond the scope of this chapter to review the entire field. Only some glimpses of basic knowledge and data of special interest to the present purpose of ultraviolet illumination will be given. There are many ex-

tensive and excellent treatises published in recent years on both the entire field and special regions of it. Exhaustive reviews are given by ELLIS, WELLS, and HEYROTH (1941), MEYER and SEITZ (1942), and DÉRIBÉRE (1947), and the volumes of DUGGAR (1936) include valuable articles on both physical and biological problems in the field. An essay on chemical aspects of light is given by BOWEN (1942). LUCKIESH (1930, 1946) has treated the field particularly from the aspects of interest here, and HARRIS (1932) has given a synopsis of the ultraviolet technology. The reviews of BÜTTNER (1938) and DE RUDDER (1938) include a number of climatological aspects, and predominantly biological reviews are given by LAURENS (1933, see also LAURENS, 1938, 1941, 1944), and BLUM (1941, see also BLUM, 1943, 1944, 1945), and therapeutic reviews by ELLINGER (1941) and KRUSEN (1941). Authoritative articles on the therapeutic applications of ultraviolet radiation are found in *Handbook of Physical Medicine*, published by the Council on Physical Medicine, U. S. A. (1945). Some excellent articles on different aspects of ultraviolet radiation are also found in the extensive volume *Medical Physics*, edited by GLASSER (1944; see the articles by BLUM; FORSYTHE and ADAMS; LAURENS; and KRUSEN and ELKINS). Some basic articles on the hygienic applications of ultraviolet as a bactericidal agent are collected in the volume *Aerobiology*, published by MOULTON (1942), and a recent review is given by HOLLAENDER (1946).

The Nature of Ultraviolet Radiation.

The special character of ultraviolet radiation as a powerful environmental factor is due to its high quantum energy. In the interaction of radiation and matter, with regard to both emission and absorption of radiation, the wave theory of radiation must be replaced, or completed, by the quantum theory in order to permit a physical interpretation of the processes. Radiation is emitted and acts as discrete packets of energy, or quanta, and the magnitude of the quantum (q) is related to the frequency of the radiation (ν) according to the law of Planck:

$$q = h \nu$$

where h is Planck's constant, which has a value of $6.6236 \cdot 10^{-27}$ erg-seconds.

The frequency (ν) and the wavelength (λ) of the radiation are related to the velocity (c) of light ($3 \cdot 10^{10}$ cm/sec.) by

$$c = \nu \cdot \lambda$$

A basic assumption in the quantum theory of radiation and hence in the whole of photochemistry and photobiology, is that of *fixed energy levels* in the atoms and molecules. An atom in its normal state contains a given amount of energy, representing the lowest level of these fixed levels; they are in some way connected with the electronic structure of the particle (atom or molecule). To change from a low energy level to a higher level the particle must absorb a quantum of energy equal to the difference in energy between the two levels. The higher levels are unstable, representing different degrees of excitation, and the highest level is the *state of ionization*, when an electron is separated from the particle. In order that ionization may occur the quantum absorbed by the atom or molecule must have a certain minimum energy.

The source of excitement may be thermal energy, electric energy or radiant energy, but it always provides the absorption of quanta which will supply the exact energy required to raise it from a low level to a high level. One way for the particle to return from the excited state to the normal state is *by emitting as radiation the same quantum of energy as was absorbed*. This is the basic process of all light-sources; when the excitement is produced by heating the emission of radiation is called *incandescence* and when produced by electric or radiant energy it is termed *luminescence* (electro-luminescence and photo-luminescence, respectively; the latter is termed fluorescence when the emission follows immediately after the excitement and phosphorescence when the emission is delayed). The tungsten-filament lamp is an incandescent radiator, while electro-luminescence is the principle of the vapour-discharge lamps such as the mercury arc, and fluorescence is utilized in the recently developed fluorescent or luminous tubes. The emission spectrum of a vapour-discharge lamp shows a number of distinct lines at different wavelengths, i. e., quantum sizes, each corresponding to the *difference* between two out of the fixed energy levels of the atom of the gas. The excitement is produced by the impact of an electron of the current passing through the vapour.

The molecules possess a great number of quantized stages, due to mutual effects between the atoms, and hence a great number of wavelengths, usually as wavelength bands or a continuous spectrum, which are emitted or absorbed. The emission is mainly produced by heating, as in incandescent lamps, or by photo-luminescence, as in fluorescent lamps.

The *absorption of radiation by molecules* is of special interest to photochemistry and photobiology. The capture of a quantum may give rise to an excited molecule, or if the absorbed quantum is sufficiently large it may produce ionization of the molecule. The excited or ionized particle is likely to start or take part in chemical reactions. Ionization obviously will be more often produced by short wavelengths (large quanta) than by long ones. Absorbed quanta of smaller size may produce fluorescence, or excited molecules, or the energy may be degraded by collisional or other processes into heat-energy (see BOWEN, 1942).

Instead of dealing with single quanta and single molecules, which make the numerical values very small, it is convenient to use the *gram molecule* ($6.023 \cdot$

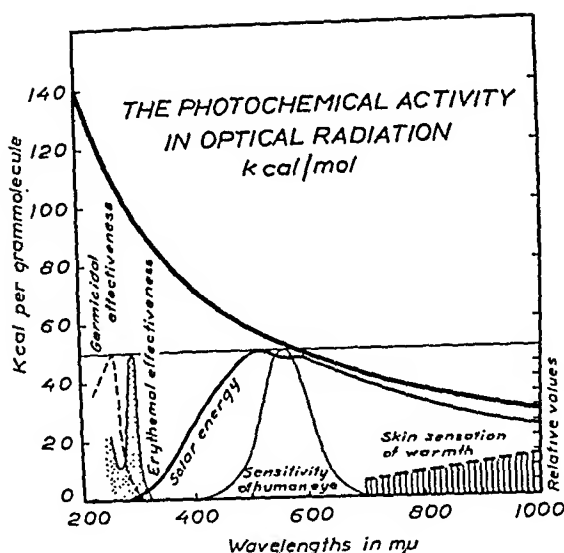


Fig. 1. The ratio of quantum energy to wavelength in the near-ultraviolet, visible, and near-infrared spectrum seen in relation to the corresponding three fundamental radiation effects on man, viz., chemical action, luminous action, and heating action.

10^{23} molecules) as the reference amount of matter. The quantum energy in a given wavelength may then be expressed in kilogram calories per gram molecule (the Einstein-unit), giving a measure of the amount of energy which would be absorbed if every molecule of a gram molecule (mole) absorbed a quantum of the frequency or wavelength in question. Thus, the magnitude of a quantum of energy of wavelength 300 μ , of frequency 10^{15} sec.⁻¹, is $6.62 \cdot 10^{-12}$ ergs, and a gram molecule number of the above quanta is equal to 95.27 kilogram calories. The energy differences in electronic transitions is about 20 to 200 k. cal. per gram molecule. To produce the same electronic excitement by heat alone would require exceedingly high temperatures.

Fig. 1 shows the relation of quantum energy to wavelength within the spectral range of interest here. Above about 760 μ the magnitude of the quanta are too small to excite even the sensitive visual substances of the retina, and the energy is degraded into heat. Due to the increasingly superficial absorption of longer infra-red wavelengths in the skin the sensation of warmth for equal amounts of radiation increases toward the long-wave infra-red. The visual substances of the eye are adapted to magnitudes of quanta corresponding to the wavelength region 400—760 μ , the visible spectrum. At the limit of about 315 μ the quanta become sufficient to produce decomposition of cellular proteins, and the same upper quantum or wavelength limit has the photo-synthesis of vitamin D. The short-wave end of the solar spectrum is situated at 295—320 μ according to the elevation of the sun above the horizon. At wavelengths below about 280 μ the lethal effects of radiation are dominant.

The long-wave ultraviolet region 315—400 μ is sometimes called UVA, the medium-wave region 280—315 μ UVB and the short-wave region below 280 μ UVC.

The Photochemical Principles.

The first act in any photochemical reaction is the absorption of a quantum of energy by an atom or molecule in the reacting system (the Equivalence Law of EINSTEIN). If every absorbed quantum results in the transformation of the absorbing molecule, the *quantum yield* of the reaction is said to be unity. This is, however, not always the case, and the yield usually varies for different magnitudes of quanta, i. e., for different wavelengths. The spectral variation of the relative efficiency factor is expressed by the *action spectrum* of the reaction. The number of molecules transformed is determined by the number of absorbed quanta times the corresponding efficiency factor of every wavelength. This is the photochemical principle underlying the use of *biologically-weighted units of radiant energy*, frequently applied in later chapters.

The yield of every wavelength is commonly but incorrectly related to the amount of energy absorbed. This amounts (Q) is expressed by the equation

$$Q = N \cdot h \cdot \nu$$

where N equals the number of quanta of the magnitude $h\nu$. According to the equivalence law the correct measure is obviously not Q but N in the equation above; the action spectra obtained by energy measurements must be corrected for relative number of quanta (BLUM, 1944). Considering the uncertainty of most of the biological action spectra this correction does not, however, add significantly to their reliability.

The equivalence law involves the two other laws inherently associated with photochemistry: the GROTHUS-DRAPER law, stating that only those wavelengths of radiation which are absorbed by a system may produce photochemical reactions in that system, and the *reciprocity law* of BUNSEN and ROSCOE, stating that the product of the duration (t) and the intensity (E) of radiant flux which will produce a given quantity of a given chemical reaction is a constant:

$$E \cdot t = k$$

Numerous photobiological processes follow this law quite accurately within certain limits of exposure times, e. g., the threshold of erythema in sunburn and the killing of bacteria. The term *dosage* is convenient to use for the product of intensity and time (Et), or, in other words, the quantity of energy applied.

Sources of Visible and Ultraviolet Radiation.

The sources of optical radiation, including the ultraviolet, visible and infra-red wavelength regions, may conveniently be divided into two main groups: thermal radiators and luminescent radiators.

The essential component in *thermal radiators* is a heated solid body, as in radiant heaters or tungsten filament lamps. Such radiation consists of quanta of a great number of sizes, and appears as a wide

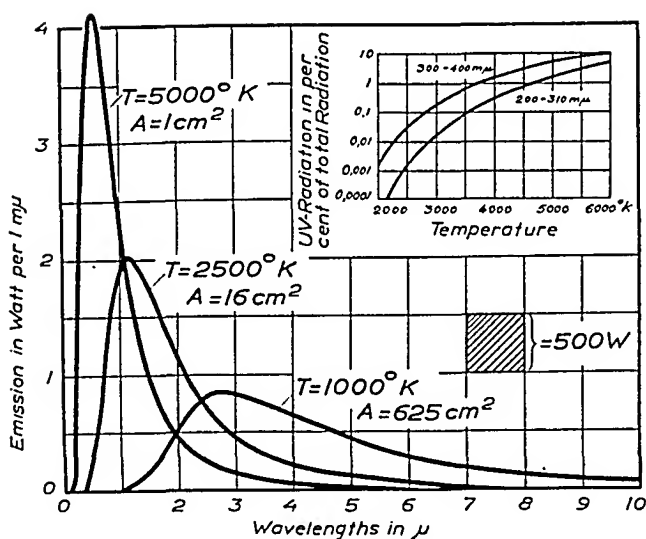


Fig. 2. Spectral energy distribution in the radiation from an ideal black body of different surface temperatures (T). The radiating surface (A) is chosen so that the total emission is similar for all cases. The inset diagram shows the percentage of ultraviolet energy for different spectral regions in the total emission at different temperatures. (After MEYER and SEITZ, 1942, and LUCKIESH, 1930.)

continuous spectrum. The region of maximum intensity of the spectrum shifts toward shorter wavelengths as the temperature of the body increases, and the body emits considerably more radiant energy as a whole. The physical law describing the spectral energy distribution as a function of temperature was formulated, for an ideal 'black body', by PLANCK in 1900; in the derivation of this formula the constant h was first found to be essential. Fig. 2 shows some curves calculated by this equation for different temperatures. The spectral energy distribution of the *tungsten-filament lamp radiation* is approximately similar to that of a black body of about $2,800^{\circ}\text{K}$, and the maximum temperature of the filament, determined by its melting point, is about $3,200^{\circ}\text{K}$. It is obvious that *artificial thermal radiators are inefficient sources of ultraviolet radiation*; the temperature of the system is not sufficient to supply energy to the molecules for the emitting of larger quanta. Only about 1/10,000th of the energy emitted by the tungsten filament is found in the wavelength region capable of producing such biological effects as sunburn and synthesis of vitamin D, and most of this radiation is absorbed by the glass bulbs of such lamps (see FORSYTHE and CHRISTISON, 1930). However, tungsten filament lamps with the bulb

of quartz or a special ultraviolet-transmitting glass have been claimed for as therapeutic sources of ultraviolet radiation ("Vitalux", "Mazda CX lamps", see, e. g., DAMMEYER and SKAUPY, 1930).

The most important source of radiation for living organisms is *the sun*. The radiation characteristics of the solar spectrum correspond approximately to that of a black body of the surface temperature $5,500^{\circ}$ K (see FORSYTHE and CHRISTISON, 1930). The relative content of ultraviolet radiation is several hundred times that of the incandescent light, but the short-wave spectral limit is found abruptly at 290—295 $m\mu$ at the surface of the earth. This limit of the spectrum is due to the absorption of shorter wavelengths during the passage of the rays through the atmosphere. The absorption of the chemically most active radiation, wavelengths below 200 $m\mu$, produces ionization of the highest layer of the atmosphere, and a formation of ozone from oxygen is induced. *The solar-formed ozone provides a very effective filter for wavelengths below 300 $m\mu$* and is the main factor determining the short-wave limit of the sun's spectrum at the surface of the earth. Thus, the solar radiation itself forms the filter in the atmosphere, which screens out the wavelengths of a chemical activity too high for permitting organic life. The maximum energy in the solar spectrum is present in the region 500—600 $m\mu$, where the sensitivity of the human eye has its maximum; an example of the rational adaptation of humans to sunlight as the paramount environmental factor.

A powerful natural source of ultraviolet radiation is furthermore *the radiation from the sky*. The scattering of light in the atmosphere is, according to the law of RAYLEIGH, inversely proportional to the fourth power of the wavelength, and hence affects the blue and ultraviolet wavelengths in the solar radiation more than the red and infra-red wavelengths. As will be discussed in a later chapter the intensity of ultraviolet radiation of wavelengths below 315 $m\mu$ on a horizontal surface fully exposed to sun and sky is more than half due to the sky radiation.

Efficient artificial sources of ultraviolet radiation are found only among the *luminescent radiators*. When light is radiated by some process in which high temperature is not the main consideration, the light is said to be due to luminescence (popularly referred to as "cold light"). The spectrum of luminescent radiation differs from that of thermal radiation in that it is always discontinuous, some-

times consisting of a group of bands, sometimes of narrow lines and, occasionally, of a combination of the two. The wavelengths of the bands or lines are characteristic of the material used. Band spectra are produced by radiating molecules, while line spectra are due to single atoms. Luminescence may be excited in various ways but ultimately these are the same in that they all involve a disturbance of the natural energy of the electrons. Of greatest interest is the *electro-luminescence* excited by the impacts in atoms of free electrons set in motion by an electric field, and the *photo-luminescence*, excited by the absorption of quanta emitted from other atoms. To the first class belongs the radiation emitted by vapours and gases in electric arcs and sparks, and the second group includes radiation by fluorescence and phosphorescence.

The *mercury vapour arc* in quartz is a particularly strong source of ultraviolet rays and is widely used for experimental studies as well as being the most common source of ultraviolet for therapeutic purposes. Quartz is used for the enclosure as it is transparent for radiation of wavelengths down to 190 m μ , while ordinary glass will transmit only rays of wavelengths longer than 320 m μ . These sources are often referred to as quartz mercury arcs, or by the misnomer "quartz lamp." Several types of such lamps are on the market. The wavelengths of emitted lines are virtually the same for all, although the relative intensities may vary to some extent. A main factor determining the relative spectral output is the *vapour-pressure of the mercury*, and hence the temperature of the arc. The common therapeutic "hot" mercury arc operates at a vapour-pressure of about one atmosphere and emits distinct lines distributed over the ultraviolet and visible region (see, e. g., RÖSSLER, 1940; ORANJE, 1943; FORSYTHE and ADAMS, 1944; COBLENTZ, 1945, 1946). Provided with an outer bulb of special glass, absorbing radiation of wavelengths shorter than about 280 m μ , such lamps are sometimes called "*sun-lamps*". Such a type is employed in the present study for the purpose of ultraviolet illumination, and will be described in later chapters.

Special types of sun-lamps were developed in the U. S. A. (GENERAL ELECTRIC) more than a decade ago, the S-1 and S-2, consisting of a tungsten filament in parallel with a small mercury arc and thus combining the thermal emission of the tungsten filament with mercury-vapour lines (FORSYTHE, BARNES, and EASLEY, 1931). The

spectral limit in the ultraviolet region is about 280 m μ , which is determined by the special glass of the bulb (see A. H. TAYLOR, 1931). Considerable amounts of light and infra-red are emitted, and the sources were previously intended also for general use as "dual purpose lighting", i. e., producing general illumination and ultraviolet irradiation at the same time (LUCKIESH, 1930; PORTER, EGELER, and STURROCK, 1932). The bulbs of the lamps last for about 200 or 300 hours of burning time and must then be replaced.

Other types of sun-lamps which in different modes combine the emission of mercury lines with thermal radiation from a tungsten filament, enclosed in the same bulb, are "the type G mercury glow lamp" and the "Ultra-Vitalux lamp." They are designed for therapeutic purposes (see MEYER and SEITZ, 1942; FORSYTHE and ADAMS, 1944).

The considerable amounts of visible radiation emitted from the mercury vapour lamps make them *powerful light-sources*, and they are in recent times widely used in combination with tungsten filament lamps for industrial lighting. The strong violet and green lines of the mercury spectrum balance the predominance of yellow and red radiation in the incandescent light with the result of a *white, daylight illumination*." The discontinuity of the spectrum may cause some disturbances in the exact evaluation of colours, but with suitable proportion of mercury and incandescent light these are usually of no practical importance (see MATTHEWS, 1940). In this modern use of mercury arc lamps for illumination no attempt is made to utilize the ultraviolet radiation for a simultaneous irradiation purpose; the quartz tube of the mercury arc is enclosed by a bulb of ordinary glass, absorbing most of the radiation of wavelengths below 315 m μ . (see p. 37).

New developments of mercury arcs are those operating at very low as well as very high mercury-vapour pressures. The *low-pressure mercury arc* ("cold quartz arc") emits most of its energy as resonance radiation of the wavelengths 253.7 and 185.0 m μ . Confined in bulbs or tubes of special glass highly transparent for wavelengths longer than 230 m μ these arcs are *efficient sources of radiation for killing bacteria and molds* and are known under trade names as Germicidal Lamps, Sterilamps, etc. Wavelengths shorter than 230 m μ must be absorbed by the enclosing glass in order to prevent excessive

The Measurement of Ultraviolet Radiation.

The measurement of radiation constitutes a basic assumption for the quantitative study of photobiological processes, but is, however, by no means a simple problem. The value of much of the published material is greatly reduced by the absence of accurate measurements and adequate identification of the spectral distribution of the radiation employed. The effects of ultraviolet radiation are highly selective so that *the measurement of ultraviolet radiation requires measurements of narrow spectral regions*; the active regions are different for different photobiological effects. The evaluation of a given radiation in regard to a given effect may be carried out in two different ways. The one is to use a measuring device with a spectral response similar to that of the effect. The other is to measure the radiation for each wavelength after having resolved its spectral components, and then to make the evaluation arithmetically with respect to the spectral efficiency curve of the biological effect. In the present study it is mainly this second procedure which has been employed. The methods will be described in a later chapter and only a brief outline of basic devices is given here. Recent reviews of radiation measurements with special respect to photobiological effects are given by, e. g., TAYLOR and HOLLADAY (1931), BRACKETT (1935, 1936), SEITZ and MEYER (1942, 1943), and HENSCHKE and SCHULZE (1942). MÖRIKOFER (1939) has reviewed the methods of meteorological radiation measurements. Recent developments in the U. S. A. are described by LUCKIESH (1946).

Receivers of radiation, used for indication or integration of quantity of radiation, are thermopiles, photoelectric devices, photo-chemical reaction systems, and the photographic plate. The *thermopiles* are uniformly sensitive throughout the spectrum and are useful as standard instruments but not very adapted to field work. The *photoelectric cells* are selective detectors of radiant energy and appropriate types may be chosen for different spectral regions. Two types are to be differentiated: the high-vacuum or gas-filled emissive type (phototubes) and the barrier-layer or self-generative type. Among the *photoemissive cells* particularly the cadmium cell, introduced by DORNO (1919), is suitable for ultraviolet measurements. By means of proper filters it is possible to provide a sensitivity curve in close agreement with the relative erythema response of human skin (see BÜTTNER, 1931). The Rentschler Ultraviolet Meter comprises special uranium, thorium, cerium, titanium and tantalum photoelectric cells connected to a glow relay tube and is used both as indicating intensity-meter and time-integrating energy-meter (RENTSCHLER, 1930). Other similar instruments are developed by A. H. TAYLOR (1934 a), who has described useful instruments for

Biological Effects of Ultraviolet Radiation.

Every biological effect of ultraviolet radiation on the human body is caused by its action on the outer membranes, mainly the skin. The effects are, however, not limited to those organs, as substances photochemically formed by the direct action on the skin are absorbed through the skin and distributed throughout the body by lymph and blood. The photobiological effects may then be suitably grouped as direct and indirect, the former including the *local changes* at the place of the absorption, and the second including the general, *systemic effects* induced in the body by the irradiation. In the scope of these pages it is impossible to give more than a brief outline of the theme, and the references must, in order to limit the bibliography, mainly be given to recent reviews and monographs.

For the design of a continuous ultraviolet irradiation, as was to be employed in the present investigation, the state of knowledge with regard to the dosage and spectral relations of irradiation and effect is of basic importance, and will be discussed in detail in a following chapter.

The Photoerythema of the Skin.

The almost immediate reddening of the skin during and after irradiation by sun or a sufficiently strong artificial source is due to the heating by the long luminous and infra-red wavelengths. The skin erythema known as sunburn (photo-erythema) differs from the similar change caused by heat in that it develops only after a *latent period* amounting to 2 hours on the average but ranging from 1 to 7 hours. Furthermore it is not as fleeting as heat erythema; the redness gradually increases and attains a maximum usually after 7 hours, and may subside after 18 to 24 hours. According to the intensity of the irradiation it may be combined with blistering and haemorrhage.

That heat is not the cause of sunburn was proved by E. P. WIDMARK in 1889. The modern era of photobiological research began with the pioneer work of NIELS R. FINSSEN, who in 1893 showed that sunburn is caused by the ultraviolet, "chemical" rays present in sunlight, and later introduced ultraviolet irradiation as treatment of the skin tuberculosis known as *lupus vulgaris* (see LOMHOLT, 1946). The present state of knowledge with regard to photoerythema is

founded in numerous works and the historical development cannot be considered here.

Using ordinary intensities, the longest wavelengths that produces erythema are between 315 and 320 m μ . The curve representing *relative effectiveness of different wavelengths* rises to a maximum at 297 m μ , descends to a minimum at 280 m μ , then rises to another maximum between 250 and 240 m μ and extends to an undertermined shorter wavelength (K. W. HAUSSER 1934; see also p. 51).

The depth of penetration of different ultraviolet wavelengths into the skin is an important factor for the interpretation of the effects. Only a very small portion of wavelengths below 300 m μ is transmitted through an ordinary thick epidermis (80—100 μ) while longer wavelengths may reach the capillary layer in considerable amounts. The horny layer of the skin shows an increasing absorption for wavelengths below 300 m μ , with a first maximum at 280 m μ (BACHEM, 1931). By regarding the corneum as a protecting screen for the Malpighian layer cells beneath it, the characteristic action spectrum of erythema may be explained as derived from the absorption spectrum of a typical protein constituent of living cells (MITCHELL, 1938). By the decomposition following this absorption it is assumed that *vaso-dilating H-substances* acting on the cutaneous capillaries are liberated, and the presence of such substances in the skin and the blood after irradiation is also proved in many different ways (see LAURENS, 1941). The latent period of the erythema is explained as due to a slowly diffusing "H-colloid." ELLINGER (see ELLINGER, 1941) has given evidence for a direct photochemical formation of histamine from histidine occurring especially for radiation about 250 m μ and being responsible for the paler and more rapidly subsiding erythema produced by these shorter wavelengths. A comprehensive, tentative scheme of the processes underlying the photoerythema is recently given by BLUM (1945) and some new additions to the theories are made by BLUM and TERUS (1946 a-b).

The importance of ultraviolet wavelengths longer than 320 m μ for the irradiation effects on the skin was first demonstrated by LOMHOLT (1936) at the Finsen Institute, who showed that the therapeutic effect of irradiation on lupus is mainly due to the spectral region 320—350 m μ and that these rays are also capable of producing erythema and, especially, pigmentation, when applied in sufficient doses. The work of I. HAUSSER (1938), extended by others (see LAU-

RENS, 1941; BLUM, 1943), has given further evidence of *specific effects of long-wave ultraviolet radiation for the pigmentation* occurring after ultraviolet irradiation. The effect of the longer ultraviolet rays is assumed to be a transformation of an uncoloured leukomelanin, present in the basal cells of epidermis, into dark melanin; the formation of leukomelanin seems to be induced by the erythema-effective wavelengths below about 320 m μ . The photochemical mechanism of the photo-pigmentation is thus to be distinguished from that of the erythema. The action spectrum of photo-pigmentation has a flat maximum in the region 340—380 m μ but extends to about 420 m μ . The transformation of leukomelanin into melanin has no latent period but requires the presence of oxygen; it is assumed to be an oxidation, and is affected by the presence of sexual hormones. The energy of radiation required for producing an immediate pigmentation without preceeding erythema by long-wave ultraviolet is of the order of 500 times that for producing erythema by the wavelength 297 m μ (cf. p. 51). A recently published action spectrum combines the erythema and tanning effectiveness of ultraviolet radiation (LUCKIESH and TAYLOR, 1939).

The *susceptibility to sunburn* may vary considerably between different individuals. There is a seasonal variation with a spring maximum, a summer minimum and a fresh rise in the autumn (see ELLINGER, 1941). Blondes are from 40 to 170 per cent more sensitive than brunettes. Endocrine influences seem to play an important part and are probably bound up with the sex function; before and during puberty the sensitivity is less than one half of that of sexual maturity age, and there is a decrease of sensitivity also in the involutionary age. Women are about 20 per cent less photo-sensitive on the year's average than men.

The most important role for the *adaptation of the skin* to erythema-effective radiation is the thickening of the epidermis, mainly its horny layer (corneum). This may be regarded as an effect of increased metabolism and growth of the skin, induced by the improved blood supply and also by the probable liberation of growth-promoting substances (see BLUM, 1943). The peeling which may follow the irradiation may be seen as a sign of this increased growth rate of the skin. The pigment appears almost exclusively in the basal cells of epidermis and is the sun-shade for the dermis as the horny layer is the sun-shade for the epidermis (MIESCHER, 1931; cf. LAURENS, 1935).

A number of other effects, chemical and physico-chemical, occurring in the skin after ultraviolet irradiation are described, as increased bactericidal properties of the lipids of the skin, increase of sulfhydryl-concentration, rise of the cholesterol content, destruction of enzymes, change of fluorescence, etc., etc., (see ELLINGER, 1941; LAURENS, 1941; BLUM, 1943). Irradiation is said to cause an intensification of the skin eruptions of epidemic diseases (smallpox, morbilli) when applied during the incubatory period (WORINGER, 1931).

Malignant tumors have been produced in experimental animals as a result of excessive exposures to ultraviolet radiation given either as sunlight or from artificial sources. The exposures needed are usually far outside the range in general use by man, either in sun bathing or in the use of rays from artificial sources. The incidence of skin cancers in white men is, however, shown to be predelicted to regions of strong sunshine, and it is significant that malignancy is rarely, if ever, seen in negroes. The effective spectral region is shown to be that of 290—334 m μ . Whether there is a specific effect of the radiation, as a formation or photodynamic activation of cancerogenic hydrocarbons, or if the malignancy is developed from chronic, precancerous lesions caused by the repeated irradiation, is still open to question (see BLUM, 1941, 1943, 1945; LAURENS, 1941; HOLLANDER, 1946). At the present state of knowledge a caution to avoid the frequent excessive exposure to artificial, and natural light seems, however, justified (BLUM, 1941).

The Photophthalmia.

The action upon the eye of ultraviolet radiation is of great practical importance. In principle it is similar to the photoerythema, i. e., dependent on a decomposition of cellular proteins. The effect is limited essentially to the cornea and conjunctiva. Depending on the dose, a latent period of several hours is followed by a more or less severe *inflammation of cornea (keratitis) and conjunctiva (conjunctivitis)* with photophobia, copious lacrimation and blepharospasm. The term photophthalmia (PARSON) is used to describe the condition, otherwise known as "snow blindness" and "flash burn" (cf. SCOBEE and GRIFFEY, 1944). The condition is often extremely painful, with sticking pains and a very unpleasant foreign body sensation, but the prognosis is favourable and the symptoms seldom last for more than a day or two.

Radiation of wavelengths about 760—390 m μ penetrates to the retina and is perceived as light. It is the lens of the eye which is chiefly responsible for stopping ultraviolet rays from reaching the retina. The absorption of the cornea begins at about 360 m μ but it transmits down to 295—300 m μ , and about the same is valid for the vitreous and aqueous humors. The absorption in the lens of

wavelengths between about 350 and 400 $m\mu$ excites a hazy bluish white fluorescence. Cataract formation by ultraviolet rays is now disputed, and the opacities are probably due chiefly to the presence of the more penetrating infra-red rays in the light sources. The aphakic eye is sensitive to wavelengths as short as 310 $m\mu$, the sensation being blue. The short-wave end of the radiation which can be transmitted to the retina is pushed toward longer wavelengths with increasing age (see, e. g., ELLINGER, 1941; ELLIS, WELLS, and HEYROTH, 1941). According to MIESCHER (1931) the cornea and conjunctiva lack the ability of adaptation to the injurious ultraviolet radiation. The rate of mitoses in the cornea is found to be influenced by ultraviolet radiation and may be used as a sensitive indicator of the radiant effects (see HOLLAENDER, 1946).

The *action spectrum* of photophthalmia is not yet well established. It was known long ago that the effect is chiefly due to wavelengths shorter than about 310 $m\mu$. Recent investigations by FISCHER, VERMEULEN, and EYMERS (1936) and COGAN and KINSEY (1946) will be discussed in the next chapter.

The Antirachitic Effect.

The discovery of the antirachitic effect of ultraviolet radiation, with its later extensions, may be regarded as *the first actual experimental evidence of the importance of sunlight as a factor of bodily health in man*. The stimulation of light research caused by this discovery is clearly demonstrated by the flood of papers which appeared subsequent to 1919 regarding the physiological effects of radiation, and particularly the relation of sunlight to rickets and the production of vitamin D in the skin (see ELLIS, WELLS, and HEYROTH, 1941).

The importance of sunlight for the etiology of rickets was first demonstrated by T. A. PALM in a paper from 1890. He employed the method of geographic survey in the study and noted that the one factor common to all districts where rickets was unknown was sunlight. This view of the etiology is now completely established. Rickets is a disease caused primarily by lack of sunlight; it is a disease of sunless areas, of winter months; a disease caused by artificial conditions which exclude sunlight, such as the massing together of the population in tenement houses and, in the East, of living in purdah (LUCE-CLAUSEN, 1938).

The clinical discovery of the therapeutic effect on rickets of irradiation with the mercury-arc quartz lamp is usually credited to HULDSCHINSKY (1919), although BUCHHOLZ apparently cured cases of human rickets with artificial light as early as in 1905. HESS and UNGER, in 1921, cured rickets with sunlight and showed that light filtered through a window of ordinary glass was ineffective and that, therefore, only the ultraviolet portion of the solar spectrum was important. The next advance was the demonstration that the light acts by a chemical mechanism. HESS and WEINSTOCK (1922) showed that a section of excised human skin after irradiation by a mercury arc when fed to rats, protected them from a deficient diet, although similar amounts of non-irradiated skin were ineffective. It was thus obvious that an antirachitic substance had been formed photochemically in the irradiated skin. In 1924 STEENBOCK and associates, and independently HESS, found that antirachitic potency could also be induced in foods by ultraviolet irradiation. The antirachitic material, which was named vitamin D by McCOLLUM in 1925, was found to belong to the sterol fraction of foodstuffs, and the provitamin was in 1927 identified with ergosterol or a hypothetical dehydrocholesterol, present as an impurity in ordinary cholesterol and phytosterols. The provitamin present in the human skin has later been identified as 7-dehydrocholesterol. The vitamin D formed by the action of the ultraviolet rays on the skin is absorbed into the blood stream. HUME, LUCAS, and SMITH (1927) proved that a protective effect could be produced by rubbing irradiated ergosterol into the skin of rachitic rabbits. The amounts of radiation necessary for the cure of the same rabbits was found to be surprisingly small; an area of skin as small as 2.5 by 3.5 cm irradiated for only ten minutes three times a week was sufficient to produce a normal calcification. Extensive studies on the antirachitic effect of artificial radiation with experimental animals were made by SCHULTZER (1927). Further investigations on the spectral and dosage dependency of the antirachitic effect of ultraviolet irradiation will be discussed in connection with the design of the ultraviolet-containing illumination system employed in the present investigation.

The definitely proved photochemical synthesis of vitamin D in the skin on irradiation with ultraviolet may be regarded as a direct effect of the radiation, while the effects induced by its absorption

into the blood lead over to *indirect irradiation effects*. There is no possibility of giving more than some fundamental data of different aspects of *vitamin D and mineral metabolism* in this survey; some of the problems will be dealt with more completely in other chapters.

Rickets is a common disease of infants and young children. It involves primarily the growing bones and the teeth and consists chiefly in a failure of calcification of regions where calcium salts are normally deposited during the growth process. This is the result of an inadequate intestinal absorption of calcium and phosphorus present in the diet because of a deficiency of vitamin D in the body. When bodily stores of vitamin D are low, the calcium and phosphorus of the foodstuffs are not absorbed but are lost in the faeces. The disease is usually accompanied by low values of either or both calcium and phosphorus in the blood serum, while the concentration of the enzyme alkaline phosphatase in the blood is increased. General signs of rickets are a catarrh of mucuous membranes, muscular weakness, increased nervous irritability and marked restlessness of the infant; it is however not sufficiently demonstrated that all of these symptoms are due to uncomplicated vitamin D deficiency (CLEMENTS, 1946). In some instances, especially when the diet is low in calcium, marked hypocalcaemia occurs, with manifestations of tetany (infantile tetany, spasmodophilia).

Modern reviews of the physiological and clinical aspects of mineral metabolism in relation to vitamin D are made by, among others, SHOHL (1939), ROMINGER (1939), GREENBERG (1939), COHN, COHN, and AUB (1942), CLEMENTS (1946) and BICKNELL and PRESCOTT (1946). The favoured current hypothesis regarding the mode of action of vitamin D in the body seems to be that vitamin D primarily acts by increasing calcium absorption in the intestine but has no, or but slight, effect on either faecal phosphorus excretion or the absorption of phosphorus, (cf. GREENBERG, 1939, 1945). But undoubtedly vitamin D exerts an action in the interior of the body also; it is well established that it increases the metabolic rate. The liberation of inorganic phosphorus from some organic phosphorus compounds seems to be promoted (RÄTHÄ et al., 1937; COHN and GREENBERG, 1939). It seems probable that vitamin D involves either directly or indirectly the parathyroid mechanism; the parathyroid glands are hypertrophied in rickets and infantile tetany (cf. LICHTWITZ, 1942). A relationship seems reasonable since the latter affects the calcium and the phosphorus metabolism and the cellular mechanism concerned with the growth and destruction of bone. An irradiation product of vitamin D, the dihydrotachysterol, may be used as a substitute for the parathormone (A. T. 10, see HOLTZ, 1939).

In a chemical sense the name vitamin D identifies a group of some ten sterol compounds, although of these only two have a practical importance. The two are alike in that they represent activated forms of naturally occurring sterols, the latter constituting the *provitamins* of vitamin D. The first is ergosterol, derived from plant sources,

which upon activation becomes vitamin D₂ or calciferol by an internal rearrangement of molecular structure. The second is 7-dehydrocholesterol, a constituent of animal lipids, and is called vitamin D₃ when it undergoes a similar change. The activation is normally effected by ultraviolet radiation of certain wavelengths; in the course of the reaction there is formed a series of compounds, one of which is the vitamin. Irradiation of sufficient intensity and duration may yield toxic substances (cf. p. 56). Cod liver oil, recommended for the cure of rickets by TROUSSEAU in 1822, consists of at least six different forms of vitamin D, the chief of these probably being the 7-dehydrocholesterol form. The ergosterol form is known as viosterol, ultranol, etc. The provitamin present in human skin is predominately 7-dehydrocholesterol, and the amount of provitamin in the skin is at least 10 and probably 100 times greater than the amount found in the inner parts of the body (see ROSENBERG, 1945).

The antirachitic principle, the vitamin D, is contained in only a few foods in the diet of an average person, namely certain oily fishes and eggs. Muscle meat including the fat, fruit, cereal and vegetables contain none or at the most only traces. The amount in milk is negligible. Liver and butter may contain a small amount but not enough to have any practical value. Summarizing it may be said that, although the diet may occasionally furnish appreciable amounts of vitamin D, most of the time it furnishes virtually none; the only proper attitude is to regard the diet as being completely devoid of the vitamin (PARK, 1940).

The prevalence of rickets is still high in many countries. FOLLIS, JACKSON, ELIOT, and PARK (1943) found by examination of bones from 230 American children between two and fourteen years of age, who died of intercurrent diseases, histological signs of rickets in about half of the material; roentgenologic symptoms were, however, found only in few cases. DUNGAL (1945) reports that 77 per cent of children of Reykjavik exhibit some sign of sustained rickets. In a broad investigation of the average health standard in northern Sweden (Västerbotten and Norrbotten) during the years 1929—1931 it was found that vitamin D deficiency was widely spread among the children (ODIN, 1934), and rachitic changes of the teeth were common (WESTIN, 1934). Clinical evidence of rickets in adults was, however, not found. LANGFELDT (1938) reports that in the northern parts of Norway the supply of vitamin D seems to be secured by the

high content of fish in the diet. In Sweden the exemplary public organization of the prophylactic health control of children up to about 3 years of age has in the last decade considerably reduced the frequency of rickets, and, in fact, cases of rickets in infants are now seldom seen here.

Systemic Effects.

The systemic effects in the body of ultraviolet radiation are primarily regarded as results of the absorption of substances formed by the direct action on the skin, e. g. histamine, H-substance and vitamin D. The penetration of ultraviolet rays into the skin is only 0.1—1 mm; the longer wavelengths may affect the minute superficial blood vessels directly, some of which are situated at a depth of only 0.3 mm (see LAURENS, 1935; ELLINGER, 1941). Reflexogenic impulses from the skin may be elicited by erythema-effective exposures (v. REIS and SJÖSTRAND, 1938).

The numerous systemic effects associated with exposure to radiation that produces sunburn are difficult to grasp and reduce to brief fundamentals. Sometimes the experimental results are extremely contradictory, which may be due to the fact that some observations have been made on sick, and others on healthy people, and the utilization of different light sources and different dosages has added to the confusion.

Among the effects of ultraviolet radiation on circulation the decrease of *blood pressure* is regarded as the most important. In healthy persons, however, the fall of systolic pressure is only about 6 mm and of diastolic pressure 8 mm of Hg (JOHNSON, POLLOCK, MAYERSON, and LAURENS, 1936), and only erythema-effective exposures are active. Carbon arc irradiation is found to be more effective than mercury arc irradiation, which is probably due to the deeper penetration of the longer wavelengths present in the former radiation. The fall of blood pressure is accompanied by an increase of cardiac output and minute volume, which lasts for several days after a single erythema exposure. The pulse may become fuller and stronger during and following a course of irradiations, correlated with increased minute volume. The effect is interpreted as the result of the spreading of vasodilating substances in the body (see LAURENS, 1935, 1938).

The effects of radiation on the blood picture and haemoglobin level have been a subject for many contradictory reports (see, e. g., ELLIS, WELLS, and HEYROTH, 1941). One reason for the variations in the results of different observers may be that the primary effect of erythema-active exposures is a vasodilatation followed by a diffusion of tissue fluid into the blood stream, resulting in dilution and increased blood volume (see MAYERSON, 1935). Another reason may be that the first effect induced is a haemolysis; it is shown that the excretion of urobilin and stercobilin increases after irradiation (TERLOV and MESHERISTKAYA, 1933; KASATKIN and BOGDANOVA, 1935). The haemolysis may stimulate to improved regeneration, but if regeneration does not occur the result will be decreased haemoglobin concentration and red blood cell count; such effects are also described.

The results obtained on irradiation of men and experimental animals with artificial sources (carbon and quartz mercury arcs) indicate very little effect on the red blood cell count and haemoglobin content when these levels are normal at the beginning of the investigations (MAYERSON, 1935; see also LAURENS, 1941). Strong, or long continued, irradiation of individuals with low initial levels results in most cases in a slight rise, seldom averaging more than 10 per cent, in the erythrocyte count, and a concomitant but smaller increase in haemoglobin which is maintained for a longer or shorter time after the irradiation is discontinued. The effect is limited and not specific and far less efficient than dietetic and drug treatment (cf. Handbook of Physical Medicine, 1945).

Long continued darkness produces no marked effect as long as the diet is satisfactory (LAURENS, 1938). "Anaemic" looking people in poorly lighted rooms, or in polar regions, etc., in spite of their pallor, often have a surprisingly high haemoglobin content (MAYERSON, 1935). Studies made on men on polar expeditions have failed to show specific effects of the long polar night. The seasonal variation of the haemoglobin content, with the highest values in summer, first reported by FINSSEN in 1894, seems to be less pronounced now (cf. ANDERSEN and NORMANN, 1948). It seems probable that other factors, nutritional or otherwise, are responsible for this variation, rather than the lack of radiation (cf. p. 115).

On the other hand, light undoubtedly exercises some effect on the *blood picture*. An increase of the reticulocyte count following

artificial irradiation and also appearing in spring has been frequently described, as well as a spring eosinophilia (see DE RUDDER, 1938). Leukocytosis, and particularly lymphocytosis, results from moderate irradiation but markedly low values in the white blood cell counts may follow excessive exposures (MAYERSON, 1935; cf. HEINILD, 1944). The results seem consistent in demonstrating a definite and sustained rise in the number of thrombocytes in man and animals on ultraviolet irradiation (MAYERSON, 1935; cf. ELLINGER, 1941).

The influence of ultraviolet radiation on metabolism offers a field containing a great number of observations. When the effects on the mineral metabolism directly or indirectly dependent on the formation of vitamin D are excluded, only a few observations may be regarded as significant.

An intensified protein breakdown is reported with increased nitrogen, sulphur and phosphorus excretion, which is considered to be a sign of the cell destruction indicated by the erythema (see ELLINGER, 1941). Both artificial and natural solar radiation causes a lowering of the blood sugar in normal men and especially in diabetics; the action is said to be similar to that of insulin inasmuch as the glycogen content of the liver and muscle increases (see LAURENS, 1941; ELLINGER, 1941). The fat and cholesterol content in blood may increase (see LAURENS, 1938).

LEHMANN and SZAKÁLL (1932 a) made an extensive study of the influence of ultraviolet irradiation on the *work and rest metabolism and the working capacity* of a group of men. Brief intense irradiation, producing an erythema, was found to lead to an increase in metabolic rate lasting for as long as 22 hours. Repeated irradiation produced a diminution of between 10 to 15 per cent in basal rate, demonstrable for 3 to 4 weeks after the last irradiation. There was a parallel increase of the respiratory quotient, which rose from 0.75—0.85 to more than 1.0. This signifies that a preferential combustion of carbohydrates follows the irradiation (cf. above). The effect on respiration was to make it easier, slower and deeper, and the pulse rate was decreased. These effects on the rest metabolism are apparently reminiscent of the effects normally occurring during a course of physical training. A marked improvement of the *working metabolism* was also found following the series of irradiations; the maximal working capacity was increased, the mechanical efficiency

markedly improved (up to 60 per cent) and the pulse rate was lowered and returned more rapidly to normal level after exercise. In a later series of the investigation, a group of men was irradiated in the same way but with the radiation filtered through ordinary window glass. None of the effects described above were then obtained; hence, the effects may not be ascribed to psychological influences, and only ultraviolet radiation of wavelengths below 320 m μ is effective (LEHMANN and SZAKÁLL, 1932 b).

The *improvement of physical fitness* indicated by these studies following on ultraviolet irradiation has been supported by later investigations. MOSCHKOWSKY (1936) found that the run of 11 km in one hour was performed with less ventilation and oxygen consumption even after the first ultraviolet irradiation; the effect lasted for about 10 days and was also produced after irradiation of only the face and shoulders. PARADE and OTTO (1939) found that the accumulation of lactic acid in the blood during work was considerably decreased after irradiation; they ascribe the effect to the activation of vitamin D which is assumed to act by means of the adrenal cortex. LEHMANN and SZAKÁLL (1944), in a repeated study, confirmed their previous results by employing different fitness tests, one of which has been applied in the present investigation also (see Chap. II). They found no effect of irradiation exposures below the erythema threshold and ascribe the effect of radiation as due to erythema substances liberated from the skin. Finally, ALLEN and CURETON (1945) studied motor and cardiovascular fitness in two groups of college freshmen, one of which received regular doses of ultraviolet irradiation. Both groups were given regular physical training. Muscle endurance tests, the Schneider test and the Schneider index rating were used for the comparison, and the experimental group showed significantly higher standard score gains than the control group in all the tests.

The influence of radiation on growth is a problem, in which relatively little well controlled work has been done on man. Studies on lower animals have, as a general rule, shown that radiation has only a negligible influence on the growth and development of mammals fed with adequate and complete diets. All results are consistent in showing that rats reared on an optimal diet grow as well in darkness as in well-lighted rooms, and that irradiation with the quartz

mercury arc has no demonstrable effect unless massive exposures are used, in which case there may be an inhibition of growth (see MAYERSON, 1935).

NYLIN (1929) studied the growth of a large number of Swedish pre-school and school children and found periodic variations during the year. The height increase was pronounced during the summer and showed two maxima: a marked maximum during March and April and a smaller but distinct maximum during November and December. The minimum height increases occurred during September and October and January and February. In general, weight increase varied inversely with height increase. Two groups of 25 boys each were irradiated at different times in winter for periods of two months or more with quartz mercury arcs supplemented with "Sol-lux" lamps for providing long-wave radiation. The average increases in height of the irradiated groups were found significantly greater than those of the controls. NYLIN attempts to correlate the periodicities in growth with the known seasonal variations in ultraviolet radiation, but it proved difficult on this basis to account for the distinct maximum in December.

Regarding, at least, the effect of ultraviolet radiation on resistance to infection a survey of the literature reveals the most contradictory opinions. Claims are persistently made for such an effect by manufacturers as well as by members of the medical profession, in spite of *most of the well controlled investigations definitely showing no effect at all*. The same is the case for vitamin D treatment (see SCHNEIDER, 1946). COLEBROOK (1929, 1946), commissioned by the British Medical Research Council, has made two authoritative large-scale investigations on artificial sunlight treatment, the first one (1929) dealing with irradiation of school children and the other (1946) with industrial and office workers (for a review of the literature see COLEBROOK, 1946). Besides the irradiated group and the control group there has also been in both trials a group receiving only the ultraviolet rays transmitted by ordinary window glass, i. e., with wavelengths below about 320 m μ screened off. In the school-children investigation, each of the three groups consisted of 100 children, and the irradiation was applied with quartz mercury arcs during the period September—March. Full erythema doses were given 3 times a week. There was no significant influence of the irra-

diation on either the weight and height increases, the incidence and duration of colds or other diseases, or on the progress of school works. HILL and LAURIE (1931) repeated her investigation, using 400 children between 9 and 12 years of age, divided into four groups, the one of which was given 2 exposures to the carbon arc weekly with doses only one tenth of those employed by COLEBROOK. By means of special clinical points, referring to weight, tonsils, conjunctiva, appetite, sleep, colds, etc., they demonstrated a definite favourable influence of the irradiation. They attributed the negative findings of COLEBROOK to "over-irradiation" of the children. An American investigation in the same years on 179 students failed completely to show any favourable effect in diminishing the annual number of colds (DOULL, HARDY, CLARK, and HERMAN, 1931). The recent trial of COLEBROOK (1946) comprised 3,000 office, factory, and colliery workers in three communities. One group received the full range of ultraviolet radiation from quartz mercury-arc lamps, given as minimum perceptible erythema doses, while another group received irradiation with the shorter ultraviolet rays screened off; a control group received no treatment at all. The results of the experiment, covering most of the winter months, give no evidence of artificial sunlight treatment being effective in reducing the incidence of sickness and accident. KOVÁCS (1947), in a discussion of these negative results, attributes them to "the fact that the minimum perceptible erythema dose is insufficient to produce therapeutic results", and recommends the use of higher dosages.

CHAPTER 3.

Hygienic Applications of Ultraviolet Radiation.

The therapeutic use of ultraviolet radiation was scientifically founded by NIELS R. FINSSEN in Denmark at the turn of the century, and was developed by, among others, BERNHARD and ROLLIER in Switzerland and Sir HENRY GAUVAIN in England. The hygienic applications of ultraviolet energy, on the other hand, arose from the discovery of its antirachitic properties and the relation to vitamin D, made in the nineteen-twenties (HULDSCHINSKY, HESS, STEEN-

BOCK, and others). An overwhelming interest developed, culminating in the nineteen-thirties. In recent years interest has again been focussed on the hygienic aspects of ultraviolet radiation and now mainly on its bactericidal properties. The pioneer of this special application of ultraviolet energy has been W. F. WELLS in the U. S. A.

Ultraviolet Radiation for Interiors.

The first trend in the early period was to utilize the daylight and sunlight content of ultraviolet energy also in the illumination of interiors. It was well-known that ordinary window glass is almost completely opaque to the antirachitic wavelengths, and in 1926 the first *ultraviolet-transmitting glass* manufactured on a large scale appeared on the market in England ("Vitaglass") and was followed by a number of other such glasses, (see ELLIS, WELLS, and HEYROTH, 1941). A simple and practical window for transmitting ultraviolet rays may be obtained by using cellophane (PFUND, 1928). The ineffectivity of such arrangements for general use was, however, soon made apparent; there is comparatively little of the valuable ultraviolet in the sunlight during the winter months, and the fraction of daylight entering a room through an ordinary sized window is very small (CLARK, 1928; KOLLATH, 1929). CLARK estimated that a person would receive as much ultraviolet radiation in two minutes in direct sunlight at noon "when out for lunch", as in an entire day at 5 meters behind a window of ultraviolet-transmitting glass exposed to the north sky. In special cases, as for balconies or sky-lighted rest rooms, ultraviolet-transmitting glass may, however, be of some value. The ultraviolet transmission of these glasses decreases considerably during use (see KOCH and WIDMARK, 1928).

considerably during use (see KOCH and WIDMARK, 1928).
 tions was the proposal to utilize *artificial ultraviolet sources for the general illumination of interiors* (LUCKIESH, 1930). Special sources were developed for this "dual-purpose lighting" at the GENERAL ELECTRIC, U. S. A. (the S-1 and S-2 sun-lamps, and others, see p. 17). Fundamental design data were presented regarding equipment to control the distribution of ultraviolet, and recommendations were made regarding the installation of such equipment for various classes of interiors (PORTER, EGELER, and STURROCK, 1932). The ultraviolet lamp was usually placed in ceiling reflectors giving di-

rect radiation, while the incandescent lamps used to produce illumination were equipped with indirect or semi-indirect units. ODAY and PORTER (1933) describe a number of such installations in different localities, as offices, hospitals, swimming pools, and other public rooms. Fixtures were arranged for the continuous burning of both sources at one time, or so that the ultraviolet or general illumination sources could be used separately. The indirect distribution of ultraviolet radiation by means of ultraviolet-reflecting paints on the ceilings was suggested as promising. It was recommended to choose the ultraviolet sources so that only very mild irradiation was produced when combined with the general illumination. The erythema response was the basis for the design, and about 1/10th of the erythema dosage was recommended as a suitable daily exposure.

The Ultraviolet Committee (No. 41) of the International Commission on Illumination has presented an extensive discussion on those exigencies which should be met by an 'ultraviolet illumination' (I. C. I., 1937, 1942), and frequent references will be given to their reports and reviews in later chapters. The present state of the technical features of producing artificial sunlight is reviewed by KREFFT (1942 a) and LUCKIESH (1946). The best practice at the present time would be a lighting installation of fluorescent lamps supplemented with mercury-arc sun-lamps (LUCKIESH), but most recent reports seem promising for the use of fluorescent "sun-tan lamps" (CLAPP and GINTHER, 1947). No experimental data, however, have appeared regarding the possible hygienic and physiological value for man of such installations for ultraviolet illumination.

The common sources for general illumination of interiors only radiate small intensities in the ultraviolet spectral range. An ordinary illumination by incandescent lamps, even with bare lamps, cannot prevent rickets in rats fed on rachitogenic diet, while a moderate level of illumination from bare mercury lamps for artificial lighting, with bulbs of ordinary glass, exerts a slight protecting and healing effect. This effect is completely spoiled if the lamps are placed in usual globes of opal glass (RONGE, 1945). Some types of fluorescent lamps have been shown to increase the calcification of the bones of chickens (WILLEGROTH and FRITZ, 1944, see FRITZ, 1945). A comparison between the radiant energy from fluorescent lamps and the radiant energy in sunlight and skylight is recently given by LUCKIESH and TAYLOR (1945).

MÖRIKOFER (1931) has made a detailed investigation on the permeability of various *clothing materials* to ultraviolet rays. The whipcord fabrics chiefly used for men's clothing are practically impermeable to ultraviolet radiation, while

materials such as cotton voile, silk stockings and cotton stockings may transmit 18—40 per cent. It is demonstrated that the weave (pore-size) is more important for ultraviolet permeability than the absorption of rays by the fibres.

Artificial Sunlight Treatments.

The collective irradiation of industrial and office workers, miners, school children, etc., is the common form for the administration of artificial sunlight treatment on a public scale. There are usually no special difficulties to arrange such treatment even for large numbers of people; the common arrangement in industries is by means of special irradiation chambers, "solaria", connected with washrooms or other sanitary facilities of the plant (see, e. g., MEYER and SEITZ, 1942). Further attention to the general hygienic potentialities of such treatments has been stimulated by the increased use of artificial lighting in workplaces and the black-out conditions during the war years.

The main interest for both the medical profession and the general public is the possibility of *promoting and maintaining health* by means of such treatments. In spite of the numbers of trials and inquiries made on this basic problem it is at present difficult to give an impartial judgement in the case; this may indicate that the possible benefits are not very pronounced and clear cut. There are at least two reasons which have made the scientific interpretation of the results so dubious. The first refers to the influences of *psychological factors*; the treatment is well-known to the public and enjoys a widespread reputation, resting on claims of both subjective and objective benefits. Both the investigator and the subjects may be prejudiced either for or against it, which may influence the interpretation. The second reason is obviously the inherent difficulties in obtaining an *objective measure of health-improvement* in an ordinary "healthy" population. Different attempts may be made to this end. The continuous record of the incidence and duration of illness, primarily colds, has been the most common method. Another estimate may be obtained by questionnaires on the subjective opinions of the effects of the treatment. A third measure of "health-improvement" may be attained by the application of special physiological or psychological tests which are likely to be related in some way with a good physical and mental condition.

In the light of these considerations the present state of the situation may be interpreted in the following way. The results regarding an effect on the *incidence and duration of illness (colds)* seem highly contradictory, but a critical review indicates very little evidence that these benefits really are produced by irradiation (COLEBROOK, 1946); anyhow, it seems to be very difficult to find the form of administration which will guarantee a favourable effect in this respect (cf. p. 35).

The opinion of the subjects themselves regarding the effects of the irradiation has been consistently in favour of the treatment; the recent trial of COLEBROOK showed that all the subjects enjoyed the treatment, and about one third of the volunteers who remained to the end of the course felt better, a few much better. The favourable impressions were in all trials prevalent among the good attenders of the course. A noticeable observation was that the subjective opinions did not differ appreciably as between the unscreened and screened lamp groups, i. e., there was no evidence of a specific effect of wavelengths below about 320 m μ . The prejudice of the subject may of course be a dominant factor for his opinion of the treatment, but some value must however be placed on it. The use of the treatment might be justified on account of subjective effects alone (COLEBROOK, 1946).

The third method stated above for approaching an objective measure on a general health-improvement, namely by means of special *physiological and psychological tests* on physical and mental condition, has not yet been applied to any great extent. The experiments of LEHMANN and SZAKALL (1932, 1944) on the effect of irradiation on working metabolism and capacity, supported by all subsequent investigators (see p. 33), may be referred to this category of tests. It seems conclusively proved that ultraviolet irradiation produces a metabolic effect in the body which appears to be similar to that produced by physical training, and is followed also by an objectively estimable improvement of physical fitness. It seems likely that the "tonic" properties generally ascribed to both natural and artificial sunlight treatments to some degree can be explained by this metabolic effect of the ultraviolet rays. Further experiments on this line seem to be a promising method of studying the general radiation effects on man.

It must be pointed out, however, that the artificial sunlight treat-

ment is only a poor substitute for natural sunbathing; the latter includes not merely a question of radiation, but of radiation, fresh air, wind, temperature, humidity, etc., and it is most often connected with pleasant and enjoying activities as water bathing and work and play in a minimum of clothing. The action of these factors in the production of benefits from an out-of-door life may not be disregarded. Sunlight is only one of the many environmental factors that affect vitality.

Disinfection by Ultraviolet Radiation.

The discovery of the lethal effect on bacteria of radiation, made in 1877 by DOWNES and BLUNT, first called attention to the importance of radiation in biology (see CLARK, 1939). Much information was collected on this effect during the early history of photobiology (see WINTERSTEIN, 1931; ELLIS, WELLS, and HEYROTH, 1941), among which may be mentioned the observation of S. BANG, made in FINSEN's laboratory 1905, that the bactericidal effect increases sharply as the wavelengths employed are decreased.

The important relation of bactericidal effect to the exposure to different wavelengths was later carefully studied by, among others, GATES (1929). The *action spectrum* obtained in this work shows a maximum around 265 $m\mu$, while the effect at wavelengths above 300 $m\mu$ was comparatively small. The reciprocity law was found to hold over a fairly wide range from a fraction of a second to several minutes exposure. The deviations occurring are most marked with young organisms, metabolically and genetically active. Practically all investigators who have determined the wavelength dependence curves for the killing of bacteria have found them to be more or less of the same shape. The sensitivity of bacteria is decreasing from the maximum at 265 $m\mu$ to a minimum around 230 $m\mu$, beyond which it rises again. The energy necessary to kill bacteria at longer wavelengths, e. g., 365 $m\mu$, is 1,000 to 10,000 times the energy necessary at the wavelength 265 $m\mu$. Some bactericidal effect is produced also by visible radiation (see HOLLAENDER, 1942, 1946; BUCHBINDER, 1942). Influenza virus is found to have about the same sensitivity curve as bacteria, while other viruses show a steadily increasing sensitivity toward shorter wavelengths (HOLLAENDER and OLIPHANT, 1944).

It is only in comparatively recent times that attempts have been made to utilize the bactericidal properties of ultraviolet radiation for the practical sterilization of foods, drugs, water and air. Among these applications the *air disinfection* has proved to be effective in reducing air-borne spread of disease, while the method has definite limitations for most of the other purposes; it is mainly restricted to surface sterilization of foods and sterilization of certain pharmaceutical preparations.

The importance of air as a vehicle for the spread of infectious agents, conceived a long time ago, was proved by W. F. WELLS in 1933 (see WELLS and WELLS, 1936, 1942). The new hypothesis developed on "*air-borne infection*" postulates that the greatest spread of respiratory infections is produced by small dried droplets floating in the air for relatively long times and distances, or by the resuspension of dried droplets in air after they have settled to surfaces such as floors, clothing, and bed clothes (see MOULTON, 1942). These "droplet nuclei" constitute the dried residues of infected droplets formed in the liquid spray from coughs and sneezes. The vulnerability of these air-borne microorganisms to ultraviolet radiation was offered as a basis for sanitary control of air-borne infections by W. F. WELLS in 1935 (see WELLS, WELLS, and WILDER, 1942). The low-pressure mercury arc generates primarily ultraviolet radiation of the wavelength 253.7 m μ (see p. 18), which is in close agreement with the maximum of sensitivity of bacteria. Enclosed in tubes or bulbs of clear ultraviolettransmitting glass it is an effective source for the radiant air-disinfection (see Council on Physical Medicine, U. S. A., 1943; COBLENTZ, 1945 a).

The general principle for the installation and operation of these bactericidal lamps for air-disinfection is the *irradiation of the air above eye-level* in the room. The intensity in the occupied zone of the room must be very small owing to the strong photophthalmia and erythema effectivity of this short-wave ultraviolet radiation. By suitable selection and installation of bactericidal fixtures the irradiated stratum of the air can be almost completely sterilized, and the local interchange of air between the upper and lower levels may provide a "sanitary ventilation" in the occupied zone with a reduction of the concentration of bacteria.

The early experiences regarding the efficiency of radiant air-disinfection in preventing the spread of respiratory diseases in different

localities, as hospital wards, children's hospitals, schools, etc., are collected in the volume *Aerobiology*, published by MOULTON (1942), together with a number of basic papers in the field. Recent experiences are briefly reviewed by HOLLAENDER (1946). It seems proved that upper-air irradiation with high intensity bactericidal lamps *is of definite value in reducing the incidence of respiratory illness in certain localities*; in a well controlled experiment carried out in a naval training centre with irradiation in the barracks the reduction of respiratory infections was estimated to be 25 per cent (WHEELER et al., 1945). It seems probable that this method of sanitary improvement will find many suitable applications.

Conclusions for the Present Investigation.

The survey given in previous chapters of the biological effects and hygienic applications of ultraviolet radiation indicates that a general, low-intensity ultraviolet irradiation of occupied rooms may possess hygienic potentialities both with respect to direct action on the occupants and bactericidal action on the air and surfaces of the room. Such an irradiation, connected with the artificial illumination system, was to be employed in the present investigation.

The general planning involves two features, in the first place, the *basic design and technical arrangement* of the ultraviolet-illumination system, and in the second place studies on the *physiological and hygienic effects* of this kind of illumination. A comparison between the artificially produced radiation climate in the experimental rooms and the *natural outdoor radiation climate* at different seasons and latitudes forms an intermediate section of the investigation.

Among the direct effects upon the occupants it is primarily the possible influences on the *mineral metabolism* and the *physical fitness* which are of interest. For both of these functions of the body ultraviolet radiation is proved to be a potential factor; to what extent it is an essential factor is not yet established. This may be evaluated by studying the influence of natural ultraviolet radiation as it is manifested by seasonal variations, and by studying the influence of an artificial supply of radiation during seasons when the natural supply is reduced.

The hygienic potentiality with respect to the bactericidal effect

of ultraviolet radiation may be studied by the *reduction of bacterial density* produced by the irradiation, and by the *reduction of respiratory, air-borne infections* which may result from this.

The extent to which these conclusions are justified may be gathered from the results of the present investigation as given in following chapters.

II.

The UV-Illumination System.

CHAPTER 4.

Dosimetric Principles and Basic Design.

For the design of an illumination system with the dual purpose of providing visual illumination and ultraviolet irradiation the prototype given *a priori* is of course the natural daylight. It is, however, of fundamental importance to realize the great difference between the ways visible radiation and ultraviolet radiation act on human beings. *The visual effect*, i. e., the seeing, is primarily related to the *intensity of light*, but in such a manner that common visual tasks can be performed at much lower intensities than those prevalent in average outdoor daylight. One of the reasons is that the response of the eye to visual stimulation is — according to the law of WEBER — approximately proportional to the logarithmic increase, or decrease, of the intensity; a depletion of the illumination from 100,000 lux in sunshine to 10,000 lux in shadow is not perceived to be greater than from 1,000 lux in indoor daylight to 100 lux in indoor artificial light, in spite of the differences being in the first case 90,000 lux and in the second case 900 lux. To this logarithmic response the great power of adaptation of the retina is further added. *The ultraviolet irradiation effects*, on the other hand, are all of slow photochemical origin and then — according to the law of BUNSEN and ROSCÖE — the *amount of radiation*, i. e., the *dosage*, is the factor determining the biological effect. A time factor is entering, as dosage equals the product of intensity and time of exposure; the surface area exposed may also sometimes be taken into account.

A consequence of this is that an approvable artificial radiation climate must not necessarily contain as much visible light as daylight in order to give satisfactory seeing conditions, but it must contain exactly the same ultraviolet intensity in order to give the same photobiological effects in the same time. A *similarity with sunlight* with regard to the relative spectral proportions of ultraviolet

and visible radiation may not be pursued, as the two components act according to different laws. The average, total sky light is in these respects a better prototype, as the ultraviolet intensity at least is as high as in direct sunlight, the light intensity however being only about 1/10th of that of sunshine (see Chap. 8).

The design of the *illumination component* of the system has thus only to be concerned with the intensity of light with respect to the visual tasks to be performed, and with some respect to the colour of the resultant light, while the *irradiation design* must include the dosage of radiation on the occupants with respect to a number of different biological responses. From this latter dosimetric point of view it is suitable to separate, at first, the following three direct effects of ultraviolet radiation on man:

the erythema and pigmentation of the skin,
the inflammation of the cornea, (photophthalmia), and
the antirachitic effect.

To these effects on the occupants an environmental effect of possible hygienic importance must be added:

the bactericidal effect.

The purpose of the design is to evaluate the spectral composition and the irradiation intensity of the ultraviolet component, which produces the effects desired but eliminates any harmful effect. To the first category must be referred, in the first place, the antirachitic effect on the occupants and the bactericidal effect on the environment, especially the air. A moderate erythematous and tanning effect of the illumination may also be considered of some value, but with regard to the small skin area exposed much systemic effect in the body is not to be expected from this. The harmful effect to be primarily avoided in the illumination is obviously the eye-irritation (photophthalmia), and the use of protecting eye-glasses may be out of question.

The experiments on the possible physiological and hygienic effects of such an ultraviolet-containing illumination (UV-illumination) were planned to be carried out on school children with the irradiation applied during their stay in the school. The classrooms were thus the localities which should be provided with the UV-illumination system. The time-table of the classes selected was almost constant for all days

and the stay of the children in the classrooms could reach about 5 hours. Artificial illumination was needed for at least 2—3 hours of the ordinary school day during the darkest winter months. From the experimental point of view it was considered suitable to apply *the daily ultraviolet exposure as high as possible*, the upper limits primarily consisting of the photophthalmia and the photoerythema. This was the actual basis for the dosimetric design, and the procedure employed to lead from this to the choice of a suitable source for the UV-illumination will be described below.

The Evaluation of Effective Radiation.

Different parts of the ultraviolet spectrum vary with regard to their efficiencies for producing the photobiological effects stated above. This wavelength dependence is expressed by the *action spectrum* of the response, and it corresponds, in the main, to the absorption spectrum of the substance responsible for the effect (law of GROTHUS and DRAPER). The degree of response is ultimately determined by the amount of active radiation absorbed during the exposure and then depends on the spectral intensities of the radiation and the time of the exposure. The best measure of the potential power which a given radiation possesses for producing a biological effect is obtained by multiplying, for each wavelength, the spectral intensity with the corresponding efficiency factor. The sum of these *weighted spectral intensities* gives the measure of the power expressed as the equivalent intensity of the reference wavelength, i. e., the wavelength of unit efficiency. This fundamental procedure to obtain a numerical measure on biologically-effective radiation may be summarized in the formula

$$E = \int_{\lambda_1}^{\lambda_2} I_{\lambda} V_{\lambda} d\lambda$$

where

E = the obtained, weighted intensity,

I_{λ} = the intensity and V_{λ} = the efficiency factor of the wavelength λ , and

λ_1 and λ_2 = the wavelength limits of the action spectrum.

The principle is further illustrated in fig. 3.

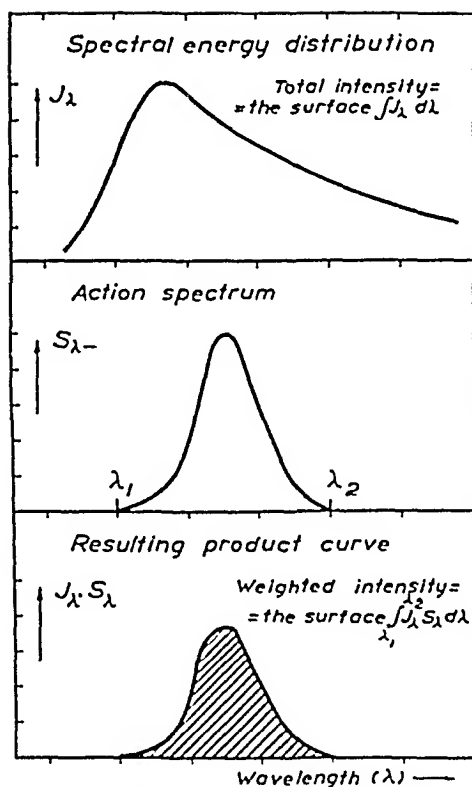


Fig. 3. The principle of evaluating radiant energy in terms of weighted units.

The physiological radiation units obtained in this way correspond in principle to the common *units for visible radiation*, i. e., lumen as the measure of luminous flux, and lux¹ as the measure of luminous intensity. The action spectrum is in this case the spectral luminosity function of the eye, with the reference wavelength 555 mμ (see fig. 1). By means of the mechanical equivalent of light, equal to 650 lumen/watt, a given luminous flux may be transformed into the equivalent flux of homogeneous radiation of the wavelength 555 mμ, measured in physical (absolute) units. In designing an irradiation system like the present one it is as suitable, or necessary to use such weighted physiological radiation units as it is to use lumen and lux in designing illumination systems.

The basic supposition for obtaining reliable measures of the biological effectivity of a radiation by such weighted units is obviously an exact knowledge of the action spectra. In this respect the best

¹ 1 foot-candle = 10.76 lux.

known is the threshold erythema response of untanned human skin, and for the antirachitic effect as well as the germicidal effect the data available permit at least a rough estimate of the spectral efficiencies. Less known, unfortunately, is the spectral response curve of the photo-inflammation of the eye. A detailed account of the data will be given below, but first the units and terms employed must be described.

The Units and Terms Employed.

A general proposal of a nomenclature and standards for biologically effective radiation was given by LUCKIESH and HOLLADAY (1931 b, 1933), who introduced the term *viton* for radiation spectrally weighted by the respective efficiency factors of each wavelength (cf. LUCKIESH, 1946). The erythema viton (E-viton) has been standardized by the Illuminating Engineering Society, U. S. A. (1933, 1935), as a term and also as a quantity equal to $10 \mu\text{W}$ of erythema flux, i. e., $10 \mu\text{W}$ of radiation after being erythemally weighted and, consequently, is equivalent to $10 \mu\text{W}$ of erythema energy of the wavelength of maximal erythema effectivity ($296.7 \text{ m}\mu$). The corresponding unit for erythema flux density (intensity) is the *Finsen*, defined as one viton per cm^2 . This system is, however, not generally adopted and many objections have been raised (SCHULZE, 1935; I. C. I., 1937, 1942). The Council on Physical Medicine, U. S. A., (see COBLENTZ, 1945), has adopted the term Erythema Unit (E. U.) of dosage intensity, equal to $10 \mu\text{W}/\text{cm}^2$ of homogeneous radiation of the wavelength $296.7 \text{ m}\mu$.

For germicidal energy LUCKIESH (1946) proposes a unit G-viton as being equivalent to one mW or μW of radiant power of maximal germicidal effectivity (stated by him to be $253.7 \text{ m}\mu$), and a Germicidal Unit (G. U.) of exposure as being equivalent in germicidal effectivity to one microwatt of the wavelength $253.7 \text{ m}\mu$ incident upon a projected area of one cm^2 for a period of one minute.

A consistent application of the idea of introducing special units as exemplified above for all the different ultraviolet irradiation effects would obviously lead to a rather complicated system. It should be emphasized that there are two different questions to be distinguished in the formulation of such biological radiation units: the one is to have a *term* for the biologically-weighted radiation, the other is to standardize a *unit* for it — with respect to both radiant flux, radiant intensity and radiant dosage. Considering the great number of different irradiation effects, each with quite different action spectra and threshold dosages, it seems reasonable to restrict the new definitions required to a term for the weighted radiation only, and not to standardize any special units for the amount of such

radiation. The quantitative data are more clearly expressed in ordinary physical units, conveniently watts or decimals of watts, and the dimension immediately gives the meaning of the datum. Thus, for instance, radiant flux must always be given in watts (or decimals thereof), radiant intensity in watts per unit area, radiant dosage in watt-seconds or watt-seconds per unit area (the latter giving the intensity of the exposure).

In the present study the terms necessary for expressing *radiant energy weighted by an action spectrum* are replaced by brief symbols, as, for instance, UVE for erythemally-weighted radiation and UVG for germicidally-weighted radiation. Erythema flux is expressed by, e. g., mW of UVE, erythema dosage by mWsec/cm² of UVE and so on. This system of nomenclature is related to the classification of the ultraviolet spectrum once proposed by COBLENTZ, by which the spectral range 400—315 mμ is termed UVA, the range 315—280 mμ UVB and the range below 280 mμ is termed UVC. This classification has been widely applied in European literature but is not generally adopted in the U. S. A. (cf. MEYER and SEITZ, 1942).

In dealing with *quantitative data of biologically-weighted radiation*, on the other hand, any possible biological unit ought to be related to the threshold dosage of the response in regard or to any other fixed degree of response. A threshold value may be expressed in, e. g., mWsec/cm² of weighted radiation, as in the case of the erythema response, the ophthalmia response, and the yield of vitamin D per unit area of skin. An expression for the power of an ultraviolet source in each of these respects may simply be obtained by dividing the weighted flux totally emitted (in mW) by the corresponding threshold value (in mWsec/cm²), the quotient cm²/sec thus giving *the area which in one second would be brought up to the threshold response if all the radiant flux were concentrated on it*. These quotients, or power-values, include the advantage of being numerically directly comparable. Thus, if the figure of the erythema power (UVE-power) is greater than that of the ophthalmia power (UVK-power) it is indicated that the irradiation of the face with unscreened eyes will produce photophthalmia before skin erythema, and in the same way the yield of vitamin D per unit area of skin may be seen in relation to the simultaneous erythema response, and so on. For an ultraviolet point source with spherical intensity distribution curve the quotient

$$\frac{4 \cdot \pi \cdot 10,000}{\text{power value}}$$

gives the time in seconds necessary for producing a threshold response at a distance of one meter from the source. The efficiency of the source with respect to different biological effects may be expressed as power-value per watt input energy.

It must, however, be strongly pointed out that the basis for such a system of biological units of radiant power is provided by two main suppositions which are not generally valid. In the first place the threshold dosage of a response is not a universal constant but varying considerably for different individuals, different skin areas, different seasons, etc. Secondly, the reciprocity law of the photobiological response is valid only within certain limits, i. e., a given dosage applied with low intensity during a long exposure may fail to produce the effect which should have been produced by the same dosage applied during a short exposure. In spite of these objections, however, this manner of biological rating of ultraviolet sources is found practicable in calculations and design of the irradiation system connected with the artificial illumination. Practical applications are given on later pages.

LATARJET (1944, see KOVÁCS, 1947) has proposed a unit sterilizing dose and a unit erythema dose (Finsen) of ultraviolet radiation, which are based on the same principles as applied here.

Dosimetric Relations of the Ultraviolet Irradiation Effects.

The Photoerythema and Photopigmentation.

The action spectrum of the photoerythema has been the subject a number of investigations. The first exact study was made by HAUSSEER and VAHLE (1921, 1928, see K. W. HAUSSEER, 1934) and their results have been supported, in the main, by the later investigations (LUCKIESH, HOLLADAY, and TAYLOR, 1930; ADAMS, BARNES, and FORSYTHE, 1931; COBLENTZ, STAIR, and HOGUE, 1932). The Illuminating Engineering Society, U. S. A., accepted (1933, 1935) an action curve based on an interpretation of the available data by COBLENTZ and STAIR (1934), and the same curve was adopted by

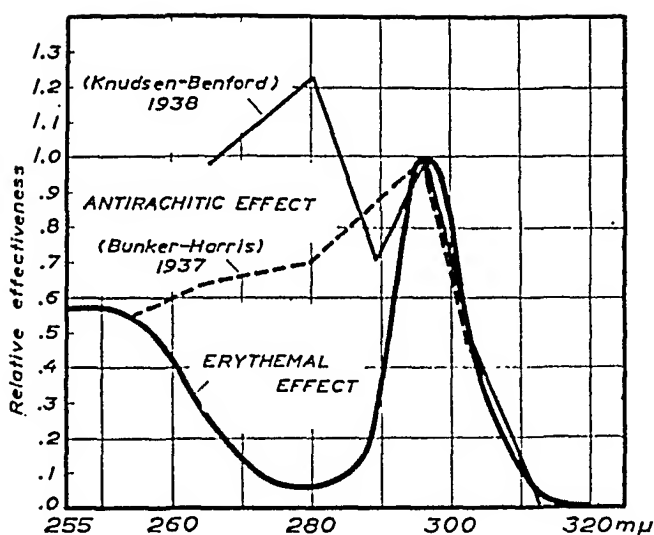


Fig. 4. Action spectra of the photoerythema and the antirachitic effect. The relative action factor for the wavelength 297 mμ is taken as unity for all spectra.

the International Commission on Illumination in 1935 (I. C. I., 1937). The curve is shown in fig. 4, and the relative erythema factors of the wavelengths of the mercury spectrum are given in Table 1. This is the curve applied in the present investigation for the evaluation of the weighted radiation termed UVE. It should be pointed out that the curve is valid only for the threshold erythema response; for higher degrees of erythema the curve is modified (ADAMS, BARNES, and FORSYTHE, 1931).

In the main, the photochemical processes underlying the erythema and pigmentation responses of the skin on ultraviolet radiation are rather well understood (for literature see Chap. 2). The erythema, inflammatory response is limited to wavelengths below about 315 mμ, while the *pigmentation (tanning) effect* is caused by the long-wave ultraviolet above 315 mμ, with the maximum efficiency in the region 340—880 mμ. A brownish pigmentation of long duration is produced by these longer wavelengths without preceeding erythema (LOMHOLT, 1936; I. HAUSSE, 1938). The direct pigmentation requires considerably more energy than the production of erythema by the shorter wavelengths; the threshold value of a pigmentation by 365 mμ is 500—1,000 times the threshold value of the erythema by 297 mμ. Thus, the *pigmentation following exposure to ultraviolet radiation mainly depends on the prevalence of high intensities of long-wave ultra-*

Table 1. *Relative Action Factors of the Mercury Spectral Lines for Producing Biological Effects.*

Effect	Ery- thema	Photophthalmia		Antirachitic effect		Germicidal effect
According to	I. C. I., 1935	COGAN and KINSEY, 1946	FISCHER, VER- MEULEN, and EYMERS, 1936	KNUDSON and BENFORD, 1938	BUNKER and HARRIS, 1937	HOLLAENDER 1942
Wavelength, mμ						
313	0.03	0.02 ¹	0.02 ¹	0.01	0.0	0.01 ¹
303	0.55	0.13	0.05	0.39	0.47	0.05 ¹
297	1.00	0.45	0.10	0.81	1.00	0.27
293	0.70	0.75 ¹	0.16 ¹	0.65 ¹	0.92 ¹	0.38 ¹
289	0.25	1.00	0.22 ¹	0.57	0.87 ¹	0.46 ¹
280	0.06	0.37	0.36 ¹	1.00	0.70	0.70
275	0.07	0.35 ¹	0.45	0.92 ¹	0.68 ¹	0.80 ¹
270	0.14	0.32 ¹	0.55 ¹	0.85 ¹	0.66 ¹	0.90 ¹
265	0.25	0.30	0.66 ¹	0.79	0.64	1.00
257	0.49	0.27 ¹	0.82 ¹		0.58 ¹	0.80 ¹
254	0.55	0.25	0.89 ¹		0.54	0.70
248	0.57	0.22 ¹	1.00			0.50 ¹
245	0.57	0.20	1.00 ¹			
Threshold dose of weighted radiation mWsec/cm ²	20 ²	20 ³	4 ³	23 ⁴	13 ⁴	0.3 ⁵ (50 mWmin/m ²)
Symbol used for weighted radiation	UVE	UVK	UVX	(UVD)		UVG

¹ Interpolated value.

² Human, unpigmented skin.

³ Rabbit eye.

⁴ Per 1 I.U. vitamin D in rat skin.

⁵ For unit lethal exposure (36.8 % survival) of *E. Coli*.

violet in the spectrum. The quartz mercury arc radiation is rather weak in the tanning-effective spectral region as compared with both sunlight and carbon arc radiation (see fig. 20, p. 89).

The threshold dosage of erythema has been estimated in connection with most of the investigations carried out on the action spectrum. HAUSSEK and GAUER (see K. W. HAUSSEK, 1934) found values about 36 mWsec/cm², COBLENTZ, STAIR, and HOGUE (1932) values about

50 mWsec/cm², and LUCKIESH, HOLLADAY, and TAYLOR (1930) give the mean threshold dosage as 4.3 mWsec/cm², all values referring to the wavelength of highest efficiency, i. e., 296.7 mμ. The Council on Physical Medicine, U. S. A., has tentatively accepted as the average minimum erythema dose an intensity of 20 μW/cm² of radiation of wavelength 296.7 mμ, acting for 15 minutes, i. e., 18 mWsec/cm² total (see BLUM, 1944). It is wellknown that the threshold dose varies considerably between different skin areas and different individuals, and there is also a pronounced seasonal variation of the skin's sensitivity (see p. 24). BLUM and TERUS (1946 b) have recently shown that the threshold dose also depends on the spectral character of the radiation in spite of its weighting by the same erythema action spectrum (that of COBLENTZ and STAIR, 1934), being about 5 mWsec/cm² of UVE for short-wave ultraviolet from the low-pressure mercury arc (predominantly the wavelength 253.7 mμ) but rising to 18.5—52.5 mWsec/cm² for ultraviolet radiation of wavelengths mainly above 280 mμ, as from the carbon arc and sun-lamps. It is evident that any average data on both the action spectrum and the threshold dosage of the photoerythema are provided with a high degree of unreliability when applied in practice; a main source of disagreement between different investigations may be the time elapsing before the observation of the response.

An important point to be mentioned here is that the reciprocity law is shown to be valid for the erythema response of the skin within wide limits — at least several hours — of the duration of the exposure (LUCKIESH, 1946; see also MEYER and SEITZ, 1942, p. 204).

The threshold dosage applied as basis for the design of the UV-illumination system in the present investigation was 20 mWsec/cm² of UVE (see Table 1). With respect to the character of "sun-lamp" of the ultraviolet sources to be used for the purpose this value may be considered reasonable.

The installation of the experimental UV-illumination system was to be made in some classrooms of a Primary and Secondary school, where the stay of the children could rise to about 5 hours per day. If the UV-illumination was on during all the lessons of the day the threshold dosage would then be reached by an UVE intensity of about 1 μW/cm² on the face and hands of the children. This figure formed the basis for the design of the irradiation component of the illumination system with respect to the erythema effect of it.

The Photophthalmia.

The prevention of ophthalmia must obviously be a most important task for the design of the UV-illumination system. Unfortunately, the dosimetric data available on this harmful irradiation effect do not permit reliable estimations of either the action spectrum or the threshold dosage on man.

FISCHER, VERMEULEN, and EYMERS (1936) first studied the spectral dependency of the "eye-injurious" effect of radiation on rabbits, using the cornea-reflectoscope as detector of the injury. The threshold doses obtained were found to decrease considerably for wavelengths below about 300 $m\mu$ (see fig. 5), being 60 mWsec/cm² at 300 $m\mu$ but only 4 mWsec/cm² at 250 $m\mu$. It seems questionable if an interpolation between the few data given in this spectral range is justified. In a recent study COGAN and KINSEY (1946) found an action spectrum of the photo-keratitis of quite a different shape, showing a pronounced peak of sensitivity for the cornea around 288 $m\mu$ with decreasing sensitivity towards shorter wavelengths. The investigation was made on rabbits and the criterion of keratitis applied was the ophthalmoscopic inspection of the cellular swelling (granulation) in the cornea. The threshold doses found by them for different wavelengths are shown in fig. 5 also. It is seen that the two investigations agree well in the region 285—300 $m\mu$ but are widely deviating at shorter wavelengths. This may be explained by the different criteria used for the threshold response. The figure also shows, that the erythema threshold is reached before the ophthalmia threshold if the short-wave limit of the radiation is situated at 290—295 $m\mu$, as in natural sunlight.

The potential ophthalmia-producing effect of short-wave ultraviolet radiation is of special interest in connection with the increasing use of low-pressure mercury lamps for air-disinfecting purposes. Up to 95 per cent of the radiation emitted by these sources consists of the wavelength 253.7 $m\mu$ (see BUTTOLPH, 1942) and thus possesses a high potentiality for eye-danger. In spite of this, comparatively little work has been done in order to establish a threshold safety dosage. ROOKS (1945) found in a self-investigation that an exposure of 3 mWsec/cm², as measured with a Rentschler photometer, was sufficient to cause a slight ophthalmia occurring 12 hours after the exposure to such a lamp equipment. This single datum obviously

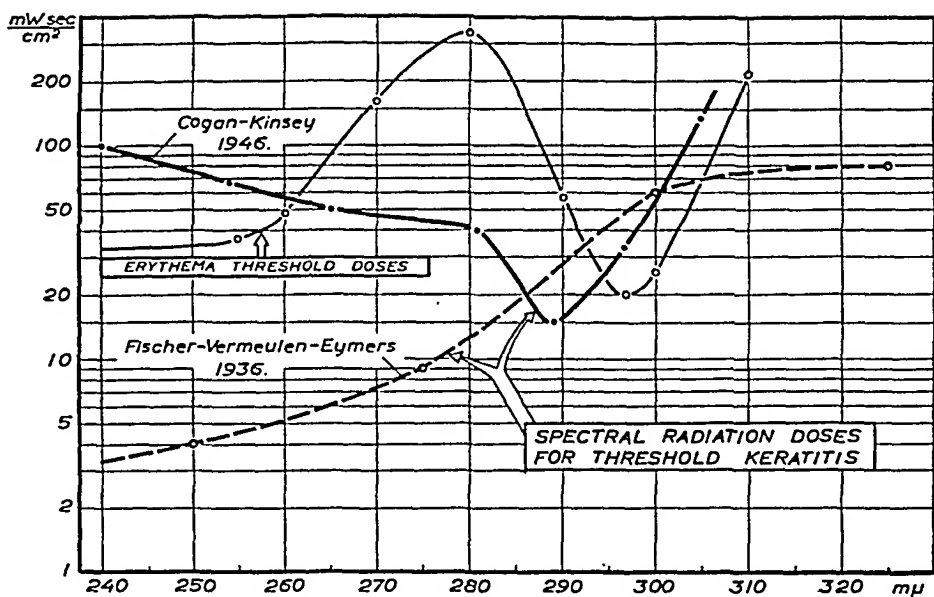


Fig. 5 Data on the threshold energy doses at different ultraviolet wavelengths for producing photoerythema and photophthalmia.

agrees better with the corresponding value found by FISCHER, VERMEULEN, and EYMERS than that by COGAN and KINSEY. *The only criterion on photophthalmia justified from the practical point of view is apparently the subjective pain and irritation*, whether this is accompanied by objective symptoms or not. It may be possible that short-wave ultraviolet rays, which are absorbed superficially in the cornea, may cause an irritation of the nerve endings there without observable granulation.

The interpretation of the action spectrum of FISCHER, VERMEULEN, and EYMERS led the International Commission on Illumination (1939) to stipulate that the intensity of radiation of wavelengths less than 280 $\text{m}\mu$ emitted by ultraviolet generators for general use may not exceed 1/10th of the energy radiated in the wavelength region 280—315 $\text{m}\mu$. The Council on Physical Medicine, U. S. A., has recently (1943) stated that the intensity on the occupants of radiation of the wavelength 253.7 $\text{m}\mu$, used for air-disinfecting purposes, may not exceed 0.5 $\mu\text{W/cm}^2$ during seven hours daily exposure, or 0.1 $\mu\text{W/cm}^2$ during constant exposure. These recommendations have been proved valuable for the safety of radiant air-disinfection installations (COBLENTZ, 1945 a). The corresponding dosage for a seven hours exposure is 12.5 mWsec/cm^2 . It should be noted that

these data are valid for the horizontal plane in the region of the occupants and that only a small fraction of this radiation actually reaches the eyes of the occupants.

In the computations of the UV-illumination system both of these action spectra have been used. Radiant energy weighted by the action spectrum of COGAN and KINSEY is termed UVK and that weighted by the action spectrum of FISCHER, VERMEULEN, and EYMERS is termed UVX. The relative action factors applied for the mercury line spectrum are seen from Table 1. The reference wavelength is for UVK 288 m μ , and for UVX 254 m μ , with the corresponding threshold dosages — according to the respective authors — 15 mWsec/cm² for UVK and 4 mWsec/cm² for UVX. The value given by COGAN and KINSEY for the wavelength 288 m μ is, as seen in fig. 5, 20 mWsec/cm²; this is valid for rabbit eyes but they state that the human eye has only about two thirds of that resistance. The corresponding intensities for a threshold response on 5 hours exposure is 0.8 μ W/cm² for UVK and 0.2 μ W/cm² for UVX.

The eye irritation symptoms sometimes occurring in persons employed in localities with fluorescent lighting were ascribed by HARMON (1944) to the ultraviolet radiation emitted from these lamps. It is, however, definitely shown that the intensities of ultraviolet radiation both above and below 315 m μ in fluorescent light are very weak and, for instance, far smaller than in northern blue skylight (LUCKIESH and TAYLOR, 1945). Such disabilities may in most cases be due to the glare from unshielded lamps.

The Photo-Synthesis of Vitamin D.

The photo-synthesis of vitamin D is regarded as the most important ultraviolet irradiation effect on man and is responsible for the anti-rachitic action, both therapeutic and preventive, of sunlight. The provitamin predominant in human skin is 7-dehydrocholesterol, which after irradiation yields vitamin D₃. The content of the provitamin is many times higher in the skin than in other organs of the body. It occurs in the lowermost cells of the horny layer and in the prickle cells of the malpighian layer and is also present in excretory products washable from the skin. The absorption of vitamin D through the skin has been actually proved (cf. Chap. 2).

It should be pointed out that the vitamin D is only an *intermediate stage* in the chain of reactions induced by the action of ultraviolet on the provitamins, the end products being antirachitically inactive

suprasterols. Over-irradiation may also yield toxic products (toxisterol, substance 248) with hypercalcaemic effects in the body. In the main, the peak of the absorption spectrum of every new substance formed in the chain of photochemical transformations is moved towards shorter wavelengths. Thus, the maximum absorption of the provitamin is found at 282 m μ , of the vitamin at 265 m μ , and of the toxisterol at 248 m μ . As the action spectrum of every transformation process may be expected to coincide with the absorption spectrum of the substance undergoing the transformation, irradiation with short-wave ultraviolet is more likely to provide over-irradiation of the vitamin than is irradiation with longer wavelengths (above 280 m μ , cf. van WIJK, 1938).

The investigations of special interest for the present purpose are those dealing with *the action spectrum and threshold dosage* of the antirachitic effect *in vivo*, i. e., by irradiating the skin of rachitic infants or animals. HESS and UNGER (1921) showed that sunlight filtered through ordinary window glass was deprived of its antirachitic ability for infants. SONNE and RECKLING (1927) found the antirachitic effect on experimental animals to be limited to the spectral range 313—239 m μ , with the highest efficiency between 302 and 253 m μ . HESS and ANDERSON (1927) found an antirachitic effect on rats, for both the range 290—313 m μ (present in solar radiation) and the range 250—280 m μ , the efficiency being higher in the latter range. In recent studies BUNKER and HARRIS (1937) and KNUDSON and BENFORD (1938) found by using large monochromators the action spectra of the threshold antirachitic effect shown in fig. 4. The data were obtained by irradiating a small area of depilated skin of rachitic rats. The relative action factors for the different wavelengths, obtained in these two investigations, are also given in Table 1. In general the curves agree well, but the maximum efficiency is found by BUNKER and HARRIS at the wavelengths 297 m μ (cf. BUNKER, HARRIS, and MOSHER, 1940) while KNUDSON and BENFORD found it at 280 m μ . The latter curve coincides closely with the absorption spectrum of ergosterol and 7-dehydrocholesterol.

An interesting point is given by KNUDSON and BENFORD, namely, that *the increase of antirachitic effect on over-exposure* differs considerably between different wavelengths. For the wavelengths 303 and 297 m μ the increase of antirachitic effect was approximately proportional to the logarithmic increase of the dosage, but for shorter

wavelengths no increase was noted, and for the wavelength 265 $m\mu$ there was an actual decrease of the antirachitic effect by exposure above the threshold dosage (= first degree of healing). The explanation is assumed to be the destructive effect of the shorter wavelengths on the vitamin (cf. above). This discovery is of interest to combine with an observation made by GORTER and SOER (1930) by irradiating rachitic infants. The therapeutic dosage of quartz mercury arc irradiation was found to be smaller than the erythema threshold dosage only when the radiation below 280 $m\mu$ was filtered away from the radiation, and the effect of the unfiltered radiation was in all cases uncertain (cf. GORTER, 1934). It seems indicated, therefore, that *the highest yield of vitamin D by irradiation of the skin is reached in the spectral range 280—313 $m\mu$, as present in sunlight*, while the shorter wavelengths may exert a destructive action overlapping the formation. A consequence of this is obviously that short-wave ultraviolet radiation, below 280 or 290 $m\mu$, ought to be depressed in the ultraviolet component of the UV-illumination system.

If the relative action factor of the wavelength 297 $m\mu$ is taken as unity for both the action spectrum found by BUNKER and HARRIS and by KNUDSON and BENFORD the relative course of the curves coincides almost perfectly with the curve of the erythema action spectrum for wavelengths above 290 $m\mu$ (see fig. 4, and Table 7, p. 96). For all ultraviolet sources of the type "sun-lamp", i. e., with the radiation below 290 or 280 $m\mu$ eliminated, the *erythemally-weighted radiation (UVE)* may then be used as a simultaneous measure of the total antirachitic efficiency of the radiation. A further assumption is of course that no weight is given to wavelengths longer than 315 $m\mu$. In the case of medium-pressure mercury-vapour sunlamps such a combination of the erythema and antirachitic units is particularly justified, as there is practically no erythema-active radiation above 313 $m\mu$ in these lamps.

Regarding the dosage of ultraviolet energy necessary for preventing and curing rickets in infants the following data from the literature may be reported. HESS and UNGER (1921) showed that irradiation with sunlight of only one wrist of a rachitic patient was sufficient to produce a general healing of the rickets. GORTER and SOER (1930, see GORTER, 1934) found that a daily dosage of 420 mWsec (0.1 Cal.) of a wavelength band around 297 $m\mu$, applied on 200 cm^2 skin area, was sufficient to cure even florid rickets. No erythema developed,

which is to be expected when considering the dosage per unit area as only 2.1 mWsec/cm^2 . That sub-erythema dosages may cure rickets was also clearly demonstrated by GERSTENBERGER and HORSH (1931), who irradiated, for 12 hours daily, practically fully clothed rachitic infants with high intensities (about 700 lux) of light from tungsten-filament lamps enclosed in bulbs of Corex D glass (Mazda CX lamps). The dosage of erythemally-weighted radiation which proved to be antirachitic was in this case about 2.4 mWsec/cm^2 per day (cf. LUCKIESH, 1946), and the skin area exposed was only that of the face and hands of the children. The figure apparently agrees well with that found by GORTER and SOER. It may thus be considered as proved that *a dosage of only about one tenth of the erythema threshold dosage applied daily on the face and hands is sufficient to secure the necessary supply of vitamin D to the infant's body.*

The yield of vitamin D per unit area of skin may be computed from the rat experiments previously cited to be about one I. U. per cm^2 per human erythema threshold dosage (see Table 1). It seems probable that the yield is considerably greater for the human skin.

If the UV-illumination for the classrooms is designed to produce a full erythema threshold exposure on the children during their daily stay in the school, this must obviously be expected to give an essential contribution to their vitamin D supply.

Bactericidal Effect of the Ultraviolet Radiation.

Regarding, at least, the bactericidal effect of the ultraviolet radiation no special design was devoted to it from the beginning. The action spectrum of this effect has a pronounced peak in the vicinity of $265 \text{ m}\mu$ (see, e. g., HOLLAENDER, 1942), i. e., in a spectral range not suitable for direct irradiation of the occupants. Toward longer wavelengths the efficiency is steadily decreasing, as shown in Table 1. Some effect may, however, be expected also for a UV-illumination with the ultraviolet radiation completely restricted to the wavelength region above 280 or $290 \text{ m}\mu$. The upper limit of the intensity is obviously governed by the direct responses of the occupants to the irradiation. As, however, the technical arrangement finally employed for the UV-illumination involved some emission of wavelengths below $280 \text{ m}\mu$, the bactericidal effect became considerable. This will be discussed more completely in Chap. 12.

The Dosimetric Design of the Illumination.

In the application of the continuous ultraviolet irradiation by means of the artificial illumination system the exposure is limited by the threshold dosage of photoerythema and photophthalmia. By the units applied here these dosages are for the erythema about 20 mWsec/cm^2 ($= 333 \text{ } \mu\text{Wmin/cm}^2$) of UVE and for the photophthalmia 15 mWsec/cm^2 of UVK or 4 mWsec/cm^2 of UVX, according to the data employed. From the interpretation given to the spectral dosimetric relations between photo-erythema and photophthalmia it is indicated that an erythema threshold exposure may be given on the face without the simultaneous production of photophthalmia if the short-wave limit of the ultraviolet radiation is situated at about $290 \text{ m}\mu$. Calculating with a maximum time for the exposure of about 5 hours daily the erythema threshold would then be reached by an UVE-intensity of about $1 \text{ } \mu\text{W/cm}^2$ on the skin of the occupants. The ophthalmia power of the lamp to be used must be small in comparison with the erythema power. *Such an exposure given by mercury arc quartz lamps provided with a suitable filter for the limitation of short-wave ultraviolet radiation would imply a high antirachitic power, and besides the direct effects on the occupants some germicidal effect would also occur on the air and furniture of the room.*

It should be pointed out that this intensity of ultraviolet on the horizontal plane in the region of the occupants may be expected to give a considerably smaller intensity actually falling on the face of the occupants owing to the oblique incidence of the rays from the ceiling equipments. Especially the eyes are effectively screened from overhead radiation in usual employment.

By using the units for the biological power of ultraviolet sources previously proposed, i. e., the quotient of total weighted flux from the source and the corresponding threshold dosage, an estimate of the lamp power required to produce a threshold exposure in a given time is simply obtained by the formula

$$\frac{P \cdot t}{A} K = 1$$

in which

P = the power-value required,

t = the time of the exposure,

A = the area to be irradiated (i. e., the floor area of the room), and
 K = the room utilization coefficient,

while 1 stands for *one threshold exposure*. This latter term may of course be chosen to the degree of exposure desired; for securing a sufficient antirachitic exposure it may be replaced by 0.1 if P stands for the UVE-power of a mercury lamp with short-wave absorbing filter.

Applied to the actual case of providing a threshold erythema exposure on school children during their stay in the classroom, with the approximate floor area 50 m² (500 000 cm²), and with an exposure time of 3—5 hours daily (\approx 15 000 sec.), and assuming the utilization coefficient to be comparatively low (about 0,20), the total erythema power required will be

$$P = \frac{500\,000}{0.20 \cdot 15,000} \approx 167.$$

Shared between four equipments each ultraviolet source should have an erythema power of about 42 cm²/sec. Considering the maintenance factor of the installation, which allows for the depletion of the ultraviolet output from the sources during the first period of use, and the smaller intensity on the face of the occupants, even a higher value would *a priori* seem applicable. On this basic assumption the experiments on the technical arrangement of the UV-illumination system were started.

CHAPTER 5.

The Technical Arrangement of the UV-Illumination System.

The technical features of the illumination system developed in the present investigation, with the simultaneous emission and distribution of ultraviolet radiation (UV) and visible light, include three special essential components:

- UV-emitting source,*
- UV-scattering reflector, and*
- UV-reflecting paint on ceilings and walls.*

Each of these components of the UV-illumination system has been a subject for special investigations and will be treated separately below. The methods employed for the physical measurements carried out are described in Chap. 7.

The UV-Source¹.

Among modern radiation sources now available, the *mercury vapour lamp* is best suited for the purpose of providing the basic source of the UV-illumination system. The mercury arc quartz lamp, operating at a vapour pressure of about one atmosphere, emits considerable amounts of energy in the short- and medium-wave ultraviolet range (see, e. g., RÖSSLER, 1940; ORANJE, 1943; FORSYTHE and ADAMS, 1944; COBLENTZ, 1945). With a suitable glass filter around the quartz burner more or less of the radiation below the wavelength 290 mμ can be absorbed (see, e. g., KREFFT, 1942 a). Such lamps are commonly called "*sun-lamps*" and with certain specifications are accepted for general use by the Council on Physical Medicine, U. S. A. (1934, 1940). A disadvantage of the medium-pressure lamp from the biological point of view is, however, the relatively small radiation output in the long-wave ultraviolet spectrum.

It is a well-known fact that mercury vapour lamps combined with incandescent lamps in special proportions give a "daylight illumination", widely used for industrial lighting (see, e. g., MATTHEWS, 1940; ORANJE, 1943; cf. p. 18). For the illumination purpose also the mercury lamp could then be expected to provide a suitable source.

The UV-source finally selected for the UV-illumination system was such a medium-pressure mercury lamp, made by the Swedish lamp factory LUMA under the trade name LK 120. The lamp, adapted for the present purpose, is shown in fig. 6. The small inner tube is made of quartz, with electrodes of a tungsten alloy. It is surrounded by a thin bulb of a molybdenum-containing glass, which strongly absorbs wavelengths shorter than about 290 mμ. The energy input, with the choke coil, is 120 watts.

The *spectral energy output* in the ultraviolet range has been deter-

¹ The sources employed were made available by LUMALAMPAN AB, Stockholm.

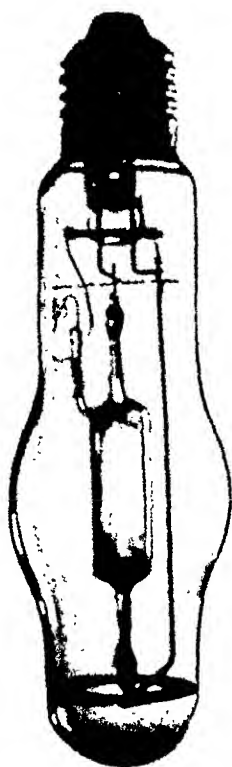


Fig. 6. The UV-source (LK 120). The small, inner mercury-arc quartz tube (the burner) is surrounded by an outer bulb (the filter) of short-wave ultra-violet-absorbing glass.

mined in several ways, as will be described later (Chap. 7). All the quantitative measurements, in absolute values, are based on comparisons with a mercury arc standard lamp ("UV-Normal"), designed by KREFFT, RÖSSLER, and RÜTTENAUER. The values given below of the total ultraviolet flux emitted per wavelength are computed with allowance for the intensity distribution curve of the ultraviolet radiation from the lamp.

In figs 7 and 8 the spectral energy distribution for the range below 350 m μ obtained by spectroradiometric comparison with the UV-Normal lamp is given for a new LK 120 lamp and for an LK 120 lamp after 400 hours of use. Fig. 19 (p. 88) gives a survey of the relative spectral energy distribution below 450 m μ , obtained by the method of direct semi-quantitative spectrography described on p. 85. Finally, Table 2 contains the values for the estimation of the erythema power (UVE), the photophthalmia power (UVK and UVX, according to the different action spectra employed) and the germicidal power (UVG) of the LK 120 lamp radiation, according to the description given in the previous chapter. Table 3 gives, for comparison, the same calculations made on the radiation data for the 250 watt

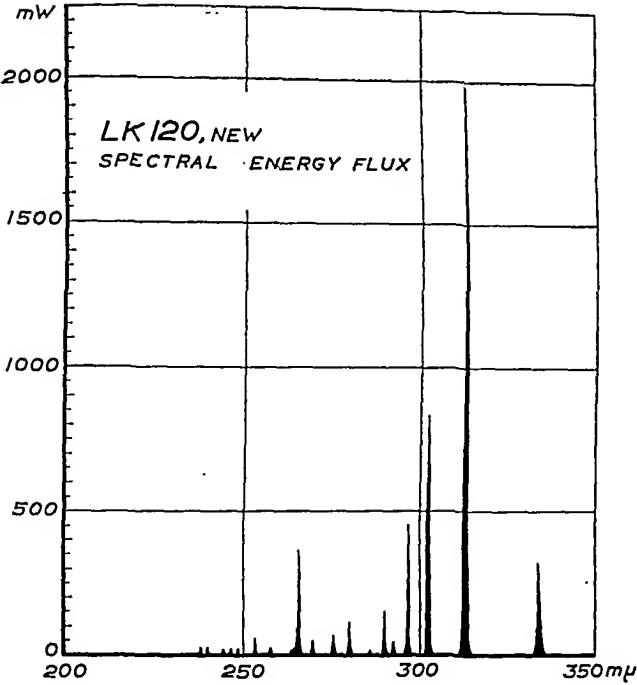


Fig. 7. Spectral energy flux in ultraviolet emitted by a new LK 120 lamp.

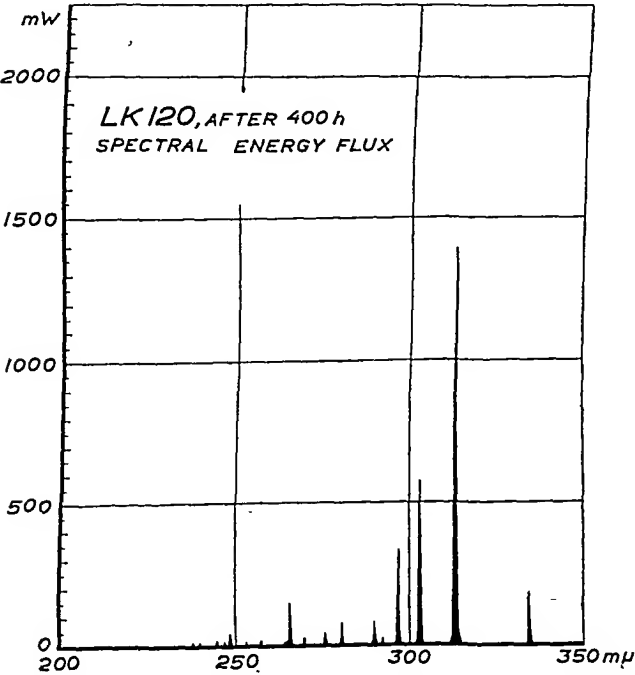


Fig. 8. Spectral energy flux of an LK 120 lamp after 400 hours of use.

Table 2. *Absolute and Weighted Radiation Data of the LK 120 Lamp.*

Wavelength, mμ	Intensity at 1 meter μW/cm ²	Absol. Flux mW	UVE mW	UVK mW	UVX mW	UVG mW
365	—	3,900	—	—	—	—
334	2.93	288	—	—	—	—
313	20.18	1,980	59	40	40	20
303	8.43	826	454	107	41	41
297	4.61	452	452	212	45	122
293	0.42	41	29	31	7	16
289	1.62	159	40	159	35	73
280	1.21	119	7	44	43	83
275	0.65	64	4	22	29	51
270	0.37	36	5	12	20	33
265	3.70	363	91	109	240	363
257	0.18	17	8	5	14	14
254	1.14	112	61	28	99	75
248	0.60	59	34	13	59	30
< 248	0.45	44	25	9	50	25
Total below 315 mμ....	43.56	4,270	1,270	790	720	946
Total below 285 mμ....	8.30	814	235	242	554	674
Weighted Power, cm ² /sec	—	—	70	79	180	3,150 (19 m ² /min)
Efficiency, cm ² /sec.watt	—	—	0.6	0.7	1.5	26

UV-Normal lamp, with the data according to RÖSSLER (1939). The upper spectrogram of fig. 20 (p. 89) shows the spectral energy distribution of this lamp found by the semi-quantitative spectrographic method. This lamp may be taken as a typical example of a common therapeutic quartz lamp.

The *absorption of short wavelengths by the outer bulb (filter)* in the LK 120 lamp is clearly seen from the tables. The emissions per watt of input energy for the spectral lines 334 and 366 mμ are about equal for both of the lamps, but for the total ultraviolet below 315 mμ the efficiency of the quartz lamp without the filter is 1.7 times higher than for the LK 120 lamp. The corresponding ratio for the UVE-efficiency is 1.6, for the UVK-efficiency 1.8, for the UVX-efficiency 3.4, and for the UVG-efficiency 2.7.

When the action spectrum of COGAN and KINSEY is used for the

Table 3. *Absolute and Weighted Radiation Data of the UV-Normal Lamp (Primary data from RÖSSLER, 1939).*

Wavelength, mμ	Intensity at 1 meter μW/cm ²	Absol. Flux mW	UVE mW	UVK mW	UVX mW	UVG mW
365	69.68	7,530	—	—	—	—
334	5.09	550	—	—	—	—
313	46.73	5,050	150	100	100	50
303	19.90	2,150	1,180	280	110	110
297	10.64	1,150	1,150	540	120	310
293	1.02	110	80	80	20	40
289	4.07	440	110	440	100	200
280	7.13	770	50	280	280	540
275	2.68	290	20	100	130	230
270	3.15	340	50	110	190	310
265	16.19	1,750	440	530	1,160	1,750
257	1.02	110	50	30	90	90
254	15.08	1,040	570	260	930	700
248	8.05	870	500	190	870	440
< 248	8.75	940	570	200	1,000	500
Total below 315 mμ....	144.41	15,010	4,920	3,140	5,100	5,270
Total below 285 mμ....	62.05	6,110	2,250	1,700	4,650	4,560
Weighted Power, cm ² /sec	—	—	246	314	1,275	17,500 (105 m ² /min)
Efficiency, cm ² /sec.watt	—	—	0.98	1.26	5.10	70

evaluation of the *ophthalmia-producing power* of the radiation (UVK), it appears that the filter does not markedly influence the ratio of erythema power to keratitis power. If, on the other hand, the action spectrum according to FISCHER, VERMEULEN, and EYMERS is employed (UVX), the eye-protecting effect of the filter is clearly indicated. Personal experience, acquired during the measurements of both types of lamps, is undoubtedly in favour of the filter diminishing the risk of eye-irritation from the same erythema-effective exposures. The same experience is also reported by VAN WIJK (1937), regarding the effect of a similar filter for the PHILIPS quartz lamp "Biosol".

The quotient UVB/UVK in the LK 120 lamp radiation is about 5, i. e., the energy emitted in the spectral range 280—320 mμ is 5 times

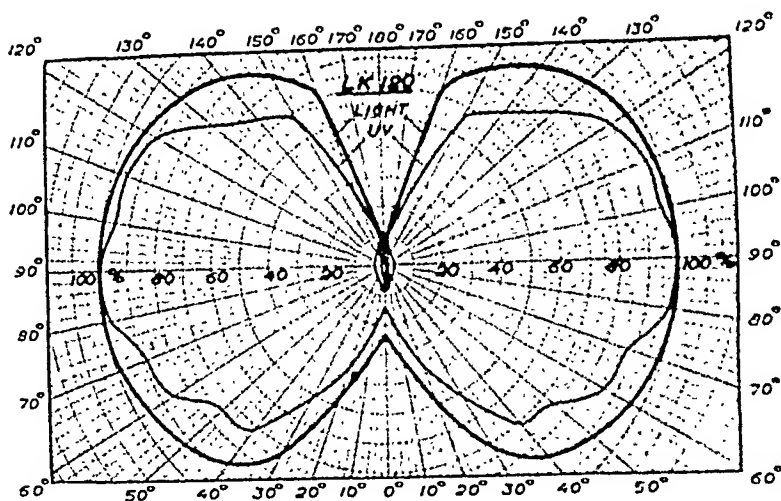


Fig. 9. Intensity distribution curves of light, and ultraviolet radiation of wavelengths below 315 $m\mu$ for the LK 120 lamp.

that below 280 $m\mu$. This ratio is approximately unity for the UV-Normal lamp radiation. The International Congress on Illumination (1935) recommended that for eye-safety this ratio should not be less than 10 for ultraviolet-containing illumination systems (I. C. I., 1937). A consequence of the comparatively strong emission of short wavelengths by the UV-source employed is the gain in *bactericidal power* (UVG). As will be shown later, the air-disinfecting effect of the UV-illumination with the LK 120 lamp as UV-source is very marked, and the risk of eye-inflammation seems to be negligible; not a single case has been reported from the experimental installations.

The *erythema power* of the lamp has also been *physiologically tested* by means of a series of increasing exposures upon normal unpigmented skin of a number of different persons. The erythema threshold for the most sensitive subjects was reached after 5–10 minutes' exposure at a distance of 50 cms from the unshielded lamp. The value computed from the spectral data given, assuming the threshold to be 20 mWsec/cm², is 7 minutes. For most of the persons longer exposures were necessary.

Some isolated experiences regarding the ophthalmia-producing power of the lamp indicate that the ophthalmia threshold is reached shortly before the erythema threshold, when the face is directly exposed to the radiation.

The *intensity distribution curves* of visible light, and ultraviolet radiation below 315 $m\mu$ for the LK 120 lamp are seen from fig. 9.

The light intensities for the different angles were measured with a colour-corrected selenium photocell (Weston Photronic cell), and the ultraviolet intensities were obtained as the difference between quartz-transmitted and glass-transmitted radiation, indicated by a selenium photocell (see p. 91). Most of the radiation, especially ultraviolet, is obviously emitted in the horizontal direction. The short-wave absorbing filter is thinner in the middle part of the lamp because of an enlargement of the bulb, and this is also shown in the distribution curve of the ultraviolet radiation.

The total *luminous output* of the LK 120 lamp was determined in the usual way by means of an integrating Ulbricht sphere photometer and colour-corrected photocell, and the average value from several lamps was 4,000 lumens. The luminous efficiency is then about 32 lumens per watt, in agreement with ordinary mercury lamps for illumination purposes.

In order to get a proper *colour of the light* in the illumination, each LK 120 lamp had to be combined in the same fixture with three 150-watt incandescent lamps, each with a luminous output of about 2,100 lumens. The ratio of incandescent luminous flux to mercury arc luminous flux is then about 1.5 : 1, which is known to be a good value in practice (MATTHEWS, 1940).

The *ratio of UVE-flux and luminous flux* of the LK 120 lamp alone is about 0.3 mW per lumen. At an illumination level of 1 lux (= 1 lumen/m²) the intensity of UVE is therefore about 0.3 mW/m², or 0.03 μ W/cm², and the UVE-intensity of 1—2 μ W/cm² required for the UV-illumination corresponds to an illumination level of 60—70 lux of the mercury-light alone. In combination with incandescent light in the proportion 1.5 : 1 the same UVE-intensity is reached by about 160 lux of illumination, which is a very reasonable value for indoor artificial illumination. These considerations demonstrated the possibility of using the LK 120 lamp as the source for the UV-illumination. The actual UVE-intensity in a room provided with UV-illumination must, however, also be evaluated with respect to the different coefficients of utilization for visible and ultraviolet radiation (see p. 76), and the estimate above is only indicative.

The radiation output of the lamp, both visible and ultraviolet, is continuously *decreasing during the time of use* (cf. SERTZ, 1939). After 400 hours, the ultraviolet output is only about one half of the initial value, as seen from fig. 8. The decrease of the luminous output is

shown by measurements to be somewhat smaller. The main reason for this uneconomical state is an obvious deposit of black vapourised electrode substance upon the inner wall of the quartz tube, but devitrification of the quartz may play some role too. The spectral transmission of the outer glass filter seems, however, not to change to any great extent, at least not during the first 400 hours of use.

Unfortunately, allowance could not be made for this deterioration to the extent desired, as the possibilities of obtaining these lamps were very restricted during the war years when the investigations were carried out.

The UV-Reflector¹.

The fitting of the UV-illumination system must distribute both the visible and the ultraviolet radiation, without the internal losses being *too great*, in order to produce a reasonably uniform illumination and irradiation over the horizontal working plane of the room. Glare from the sources must also be avoided.

Ordinary glass of some millimeters thickness, as used in most luminaires for artificial lighting, completely absorbs ultraviolet radiation of wavelengths shorter than about $315\text{ m}\mu$ (see, e. g., KOCH and WIDMARK, 1928). The ultraviolet-transmitting glasses now available are still *too expensive* for general use in luminaires.

Hence the fixture finally adopted for the UV-illumination system was of the *reflector type*, consisting of vertical concentric rings of aluminium, open upwards and downwards, as shown in the photograph of fig. 10. The inside surfaces of the rings were chemically etched in order to give a diffuse reflection throughout the visible and ultraviolet spectrum (see TAYLOR and EDWARDS, 1931). The reflection factor of the treated surface for the wavelength $297\text{ m}\mu$ was spectroradiometrically determined to be 70 per cent, which is in agreement with the data given by TAYLOR and EDWARDS for this kind of surface treatment. The outer surfaces of the rings were painted with a *special ultraviolet-reflecting paint*, described below. In the bottom of the lowest ring of the reflector a diffusing opal glass plate could

¹ The design of the reflector was made in collaboration with G. HASSEL, Swedish Lighting Development Society, Stockholm. The reflectors were manufactured by ASEA Electric Ltd., Stockholm.

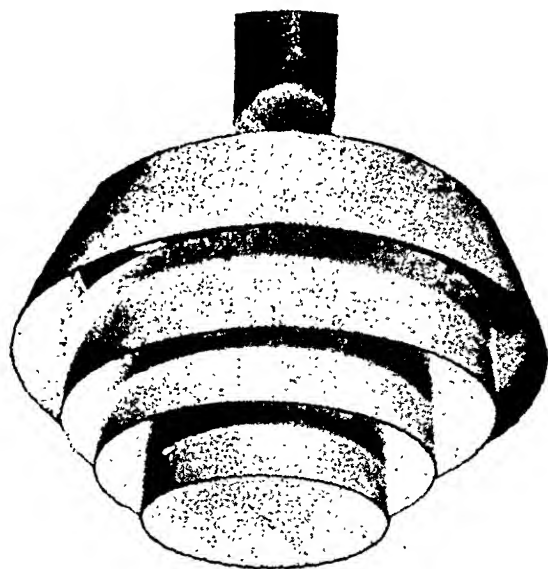


Fig. 10. The UV-reflector. The concentric rings are made of aluminium. The inside surfaces are chemically etched and the outside surfaces are painted with an ultraviolet-reflecting paint. One LK 120 lamp and three 150-watt incandescent lamps are placed in the reflector.

be placed, in order to diminish the ultraviolet emission from the equipment.

The mercury lamp LK 120 was placed in the centre of the reflector, surrounded by the three incandescent lamps. The heights and relative positions of the rings were so arranged that the bright mercury lamp could be seen only from a point just below the fixture and so that the glare from the other lamps was also reasonably avoided. With the opal glass plate situated in the lowest ring the mercury lamp was completely invisible from below. The choke coil of the mercury lamp was placed in a ceiling box.

The *intensity distribution curves* of visible light, and ultraviolet radiation of wavelengths below $315\text{ m}\mu$ from the reflector, with and without the opal glass plate, are shown in fig. 11. The visible radiation is distributed upwards and downwards to approximately the same extent, while the ultraviolet distribution is predominantly indirect, especially when the opal glass plate is in position. The horizontal component of the distribution curve is obviously almost completely absent for both the visible and the ultraviolet radiation.

The *efficiency of the reflector* with respect to the *luminous output* was found to be 57 per cent, and with respect to the light from the central mercury lamp only it was 54 per cent. These luminous efficiency determinations were made in the usual way with the aid of a large Ulbricht sphere photometer. This method was not applicable

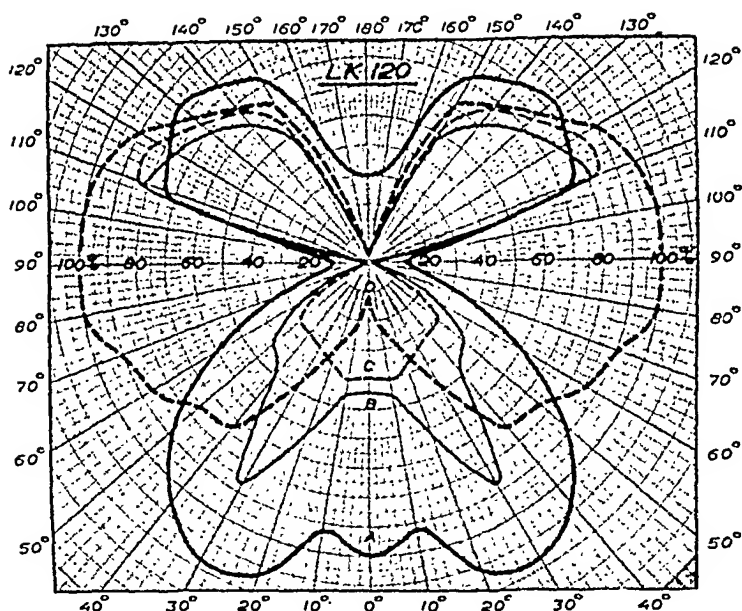


Fig. 11. Intensity distribution curves of the UV-reflector. A = light distribution, B = ultraviolet distribution from the reflector without opal glass plate, C = ultraviolet distribution with opal glass plate, D = ultraviolet distribution from the bare LK 120 lamp.

for determinations of the total ultraviolet efficiency of the reflector, as the paint on the inside of the sphere was found to absorb the ultraviolet radiation. The *ultraviolet efficiency* of the reflector was then computed from the intensity distribution curve of the ultraviolet radiation (see p. 84). This computation was made in such a manner that the component efficiencies of the direct and indirect radiation were also obtained. The values are given in Table 4. It is seen that the opal glass plate in the bottom of the reflector diminishes the efficiency of the direct ultraviolet component by one half. The component efficiencies are later used for the calculation of room utilization factors.

Table 4. *Efficiency of the Reflector for Ultraviolet below 315 m μ .*

Reflector	Direct component	Indirect component	Total ¹
Without opal glass....	0.20	0.28	0.48
With opal glass.....	0.10	0.32	0.42

¹ The horizontal component is neglected.

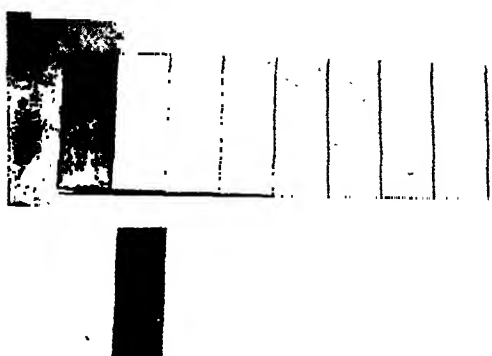


Fig. 12. Ultraviolet reflection photographs of various paints; the upper series for ordinary light, the lower series for ultraviolet radiation. From the left: unpainted wooden plate; barium sulphate in casein (the UVR-paint); barium sulphate in oil; the rest, zinc oxide and titanium oxide in oils.

As the ultraviolet reflection factors of the ring surfaces, especially the external painted surfaces, decrease towards shorter wavelengths, the efficiency values given must be considered as averages. For wavelengths below 290 $m\mu$ they are probably somewhat smaller.

The UV-Reflecting Paints.¹

Most common paints for indoor use, such as oil paints and those containing zinc oxide or titanium oxide as pigment, have a negligible or very small reflecting power in the ultraviolet spectral range (see LUCKIESH and HOLLADAY, 1931 a; A. H. TAYLOR, 1934 b). This may be clearly illustrated by *ultraviolet photography* (fig. 12). Some common mixtures were painted on wooden plates and photographed, both in the usual way, with a yellow filter and panchromatic film (the upper series), and also with a special "UV-camera", equipped with UV-filter and quartz lenses (the lower series). The UV-filter used was a silvered quartz plate with transmission only in the range 310—330 $m\mu$, combined with the SCHOTT filter UG 5 (see also p. 90.)

To install a UV-illumination system in a room with no reflection of the ultraviolet rays by the ceiling and walls would obviously be as ineffective and uneconomical as to install a common lighting system in a room with black or very dark ceiling and walls.

¹ The preliminary experiments were carried out in cooperation with T. JOHANSSON, Military Research Institute, Department I, Stockholm. The definite composition was made in cooperation with FERNISS AB FERBO, Stockholm, who also manufactured the paints.

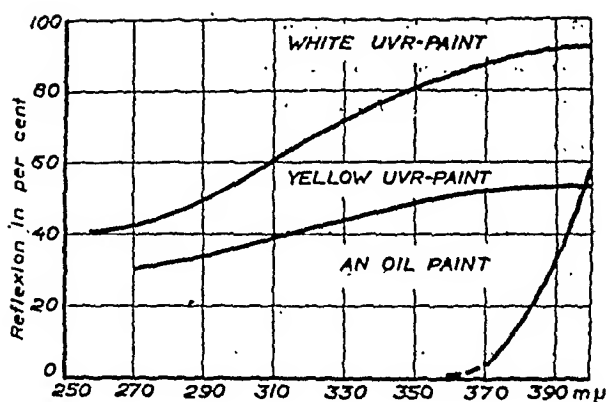


Fig. 13. Spectral reflection in ultraviolet of the UVR-paints compared with an ordinary oil paint.

The investigation of the factors determining the UV-reflecting ability of a paint leads to the conclusion that the *paint pigment* must have good UV-reflecting power, and the *paint binder* good UV-transmitting power. Different paints with improved UV-reflecting power were described by LUCKIESH and HOLLADAY (1931), and other such paints were recently studied by WILLCOCK and SOLLER (1940). As binder for the paint, casein or nitrocellulose lacquer has mostly been used, and the pigment has in most cases been magnesium carbonate. A survey of this field was recently given by DÉRIBÉRE (1947), who also points out the possibility of using wall-papers for ultra-violet-reflecting purposes.

The experiments carried out during this investigation resulted in the discovery that a good UV-reflecting paint could be obtained by using *barium sulphate* as pigment and a *casein solution* as binder. With these intentions the Swedish paint factory FERNISS AB FERBO has made two useful UV-reflecting paints ("UVR-paints"), one white and the other with a slightly yellow tint. The spectral reflection measurements of these paints have been made spectroradiometrically, using magnesium oxide as a standard (see p. 90), with the results shown in fig. 13.

During filter measurements made with the UV-Normal mercury lamp placed in a special Ulbricht sphere (p. 81), which had the inside surface freshly painted with the white UVR-paint, it was noted that the reflection factor in the spectral range 280—320 mμ decreased exponentially to about 80 per cent of the initial value during the first hours of the strong irradiation. This is probably due to a photoche-

mical influence upon the casein binder. The same observation was also made by WILLCOCK and SOLLER on some similar paints.

A disadvantage of these two UVR-paints is that they are not quite resistant to water, and therefore not washable. For use in the UV-reflectors it was considered necessary to have a washable paint. The laboratory of the FERBO paint factory was also successful in making such a paint with approved ultraviolet reflection for this purpose. The reflection factor in the range around 300 m μ was, however, not higher than about 30 per cent. This is the main reason why the efficiency of the reflector for ultraviolet was lower than for visible light.

CHAPTER 6.

The Radiation Climate of the Experimental Rooms.

The Experimental Rooms Selected.

For the purpose of studying the hygienic and physiological effects of the UV-illumination two ordinary class rooms of a large Secondary school in Uppsala, and the single room of a small Primary school situated in the outskirts of the town, were equipped with the UV-illumination system. In each of the Secondary school rooms, both with a floor area of 55 m² and a ceiling height of 3.55 m, the walls and ceilings were painted with the white UVR-paint, and four of the UV-equipments were installed at a height of 2.7 m (see fig. 14). The Primary school room was somewhat smaller, with a floor area of 45 m² and a ceiling height of only 2.7 m. The ceiling was painted with the white UVR-paint, the walls with the yellow UVR-paint. Here also four UV-equipments were installed, 2.1 m above the floor.

Theoretical Estimation of the Radiation Intensities.

From the dimensional data of the room, the radiation data of the sources and the efficiency data of the equipments given above, the intensities of ultraviolet energy upon the working planes of the rooms were calculated in the same way as is recommended for accurate de-



Fig. 14. One of the experimental classrooms in the Secondary school, equipped with UV-illumination.

signs of interior illumination, i. e., by means of the 'three-curves' method of HARRISON and ANDERSON, as recently described by BOAST (1942). The accuracy of this method is said to be better than 10 per cent.

The principle of the method is that the radiation from the equipments is divided into three components, a direct one (D), a horizontal (H), and an indirect (I), which are treated separately and finally added. For each component the coefficient of distribution (C), giving the ratio of the flux received at the working plane to that emitted by the equipment, is obtained from average nomographic charts, with allowances for the room index as a measure of the shape of the room, and for the wall and ceiling reflection factors. The room index is also taken from a nomographic chart, and is determined by the ratio of the ceiling or mounting height to the room width, and the ratio of the room length to the room width. To get the coefficient of utilization (K), of the complete system, the distribution coefficients (C) are weighted against the respective efficiencies (η) of the equipment, i. e.,

$$K = C_D \eta_D + C_H \eta_H + C_I \eta_I.$$

In the case of the ultraviolet radiation from the actual equipments, the horizontal radiation component ($C_H \eta_H$) is negligible.

Besides the coefficient of utilization, a *maintenance factor* (M) must also be introduced, allowing for the diminution of the radiation intensities with the ageing of the sources and also for the accumulation of dust and dirt on the equipments and the reflecting surfaces. Then, the density of the radiant flux (E) on the horizontal working plane, assumed to be 0.9 m above the floor, is computed from the general formula

$$E = \frac{n F K M}{A}$$

where A equals the room area and n the no. of sources, each with the radiation flux F .

For the installations in the two larger, Secondary school rooms, the coefficient of utilization (K) for the *ultraviolet radiation* was found to be 0.24, i. e., 24 per cent of the ultraviolet radiation emitted by the UV-sources is received on the working plane. With the ultraviolet absorbing opal glass plates placed in the reflectors, the value of K was diminished to 0.17. In the smaller, Primary school room the values were 0.25 and 0.18 respectively. The maintenance factor (M) was, by analogy, estimated to be 0.70, provided that the UV-sources were exchanged after about 400 hours of use. The initial intensities are of course obtained by putting M equal to unity.

The coefficient of utilization of the illumination system with respect to the *visible radiation*, i. e., the light, was found to be about 0.35 for all the rooms, with only a slight reduction when the opal glass plates were in the equipments.

Table 5 gives the intensities in the experimental school rooms of the different spectral radiation components of interest, obtained by this method of calculation. *It gives a synopsis of the radiation climate, to which the children were exposed during the investigation.* The approximate threshold time values given are strictly valid only for horizontally exposed surfaces. Actual measurements indicated that the intensities on vertical surfaces in the work region were about 30—50 per cent lower, with correspondingly higher values of the time for threshold exposures.

For the purpose of the medical investigation it was stated that *the illumination should be in use during at least three lessons daily (about 150 minutes)* and more if necessary. During the darkest winter months the artificial illumination was normally in use for all the lessons (about 300 minutes).

Table 5. *Intensities of Visible and Ultraviolet Radiation in the Experimental Classrooms.*

Component of Radiation	Secondary School Rooms		Primary School Room	
	Intensity at the horizontal working plane	Approx. time for threshold exposure	Intensity at the horizontal working plane	Approx. time for threshold exposure
Light	270—300 lux	—	400—500 lux	—
UV 400—315 mμ	5 μW/cm ²	—	6.7 μW/cm ²	—
UV below 315 mμ	5.1 »	—	7.0 »	—
UV below 285 mμ	1 »	—	1.3 »	—
UVE	1.5 »	220 min.	2.0 »	160 min.
UVK	0.9 »	190 »	1.2 »	145 »
UVX	0.8 »	85 »	1.1 »	65 »
UVG	1.1 »	20 » ¹	1.3 »	17 » ¹

Opal glass screens in equipments: 30 per cent lower intensities.

Initial conditions: 40 per cent higher intensities.

¹ For about 99 per cent killing of *E. coli* (40 % rel. humidity). See Chap. 12.

If the intensity of the visible radiant energy in the experimental rooms (300 lux) is transformed to the equivalent absolute intensity of the reference wavelength in the luminosity curve (555 mμ), by means of the mechanical equivalent of light (= 1.5 mW per lumen), the value 45 μW/cm² is obtained. The intensities of weighted erythema radiation (1.5 μW of UVE) in the experimental rooms are then biologically comparable with about 10 lux of illumination. This gives an idea of the intensity relation of effective visible, and effective erythema-producing radiation.

Experimental Controls of the Radiation Intensities.

The intensity values computed have been checked in physical as well as physiological ways. With a *photoelectric filter-difference device* (see p. 91) indicating only the radiation below 315 mμ, a reading was taken of the intensity one meter away from the bare UV-source (LK 120), and then at twenty places in one of the Secondary school rooms, with the illumination on. By assuming the intensity of radiation below 315 mμ at one meter from the LK 120 to be 44 μW/cm², as determined by other measurements (Table 2), the intensities in the school room measured in this way were found to vary from 3.2 to 9.4 μW/cm², with an average of 6.3 μW/cm². The correspond-

ing intensity calculated for a new installation was $7.3 \mu\text{W}/\text{cm}^2$ (Table 5), with respect to the maintenance factor $5.1 \mu\text{W}/\text{cm}^2$. No attempt has been made to correct these readings for the influence of the small change in spectral composition of the radiation due to the reflection from the ceiling and walls. The measurements were made with new LK 120 lamps, but after the installation had been in use for one year.

The *physiological test* employed has been the development of erythema. It was found that in the larger Secondary school rooms where the erythema threshold exposure should be reached after 220 minutes, the children did not show any skin irritation after the daily three hours of exposure. The teacher, who sometimes had to work there for longer times in the day, felt a slight hotness in the face after about five hours in the illuminated room. Similar experiences were also obtained by other subjects. In the Primary school room, with the higher intensities, *all the children had slight but definite yellowish-brown pigmentation of face and hands* after the first weeks of the experiment. The pigmentation of the children is obviously a sign of adaptation to the ultraviolet radiation.

At the beginning of the experiment the opal glass plates were used in the reflectors, as the calculated intensities, especially of the ophthalmia-effective radiation, seemed to be too high. The plates were taken away one by one during the first week, without any injurious effect. The intensities were then about 40 per cent higher than indicated in the table above. The Council on Physical Medicine, U. S. A. has stated (1943) that the intensity of short-wave bactericidal ultraviolet radiation in a room must not exceed $0.5 \mu\text{W}/\text{cm}^2$ on the occupants when the exposure is 7 hours, and $0.1 \mu\text{W}/\text{cm}^2$ when the exposure is 24 hours. These safety intensities are calculated on the basis of the erythema response (cf. p. 55).

Experiences from other similar installations of UV-illumination, with about the same intensities as in the school rooms (a textile factory, and personal laboratories at the Institute), indicate that even eight hours of exposure, and probably more, to the illumination do not cause more than a slight or moderate erythema, to which the skin is rapidly adapted, but *without any eye discomfort. No single case of this has hitherto been reported from the different installations made.*

The main reason why the intensity of the ophthalmia-effective radiation can be kept at such comparatively high values without immediate risk of eye irritation, is certainly that in ordinary employment

the eyes are very effectively screened from the overhead radiation. The reflection from the lower parts of a room for the short-wave ultraviolet radiation is in most cases negligible. It should be noted, however, that ordinary book and writing paper reflect 20—40 per cent of radiation around 300 m μ (see A. H. TAYLOR, 1934 b). In the case of a hospital room, with patients lying in beds, the risk of photophthalmia from such a UV-illumination would probably be considerably higher.

An important advantage of the prevalent short-wave ultraviolet radiation, despite the danger of latent eye-injury, is *the marked bactericidal effect of the illumination throughout the room*. The interpretation of this will be given in Chap. 12. An unfortunate effect, on the other hand, is the deleterious action on most flowers and plants in the room, which seems unavoidable.

The *illumination level* (300—500 lux) in the school rooms equipped with UV-illumination systems was considerably higher than is usual in Swedish classrooms. Some glare from the reflectors was inevitable, but in general the installations became highly valued by both teachers and children. The colour of the light was pleasant, being reminiscent of natural sunlight.

To sum up it may be said, that *the exposures of ultraviolet energy in the Secondary school rooms were just below the erythema threshold, and in the Primary school room just above this threshold*. No case of discomfort from the eyes or otherwise has been reported, in spite of the comparatively high intensity of short-wave, strongly bactericidal radiation present in the illumination.

CHAPTER 7.

The Physical Methods Employed.

The development of the UV-illumination system described in the last chapters has obviously required a number of special physical and technical measurements. The methods employed have generally been rather primitive, because of the lack of suitable instruments. For instance, the only photoelectric devices available have been selenium barrier-layer photocells, which also had to be used where the

special phototubes made for ultraviolet radiation would have been more suitable. The lack of a sensitive, calibrated thermoelectric device has made all the quantitative radiation measurements, in absolute units, dependent upon the constancy of a standard lamp for ultraviolet radiation.

Special attention should be drawn here to the *semi-quantitative spectrographic method* described on page 85, which has been developed during the present investigation.

Measurements of Spectral Energy Distribution.

The UV-Normal Lamp.

All the measurements of spectral intensities and spectral energy distributions have been based upon comparisons with the *UV-Normal lamp*, described by KREFFT, RÖSSLER, and RÜTTENAUER (1937), and previously made by OSRAM A. G., Berlin. This lamp is a medium-pressure mercury arc quartz lamp of 250 watts, D. C., and is said to give a constant spectral energy output, within the limits ± 5 per cent, during 200 hours of use, when burning under certain given conditions. Spectral data for the lamp, in absolute units, are given by FISCHER (1937), RÖSSLER (1939) and WEISSFLOG (1940). There are some differences between the results of these measurements, but for the range 315—250 $m\mu$, which is of greatest interest in this investigation, the data given by the different authors are fairly uniform. The constancy of the lamp is supported by WEISSFLOG.

The lamp available for this investigation was received direct from OSRAM (Berlin), and has only been used for this work. The spectral data given by RÖSSLER have been used as standard values (Table 3, p. 66). It has not been possible to check these values further.

The lamp is run on direct current from a battery of about 200 volts, and is in the vertical position with the anode downwards. The arc is started by means of a Tesla inductor or other ionization device. After the starting period the input power of the lamp is adjusted to exactly 250 watts by means of a resistance. The arc voltage is then about 130 volts. A complete description of the use of the lamp is given by KREFFT (1942 b).



Fig. 15. The Ulbricht sphere photometer with filter and photocell equipment for measuring ultraviolet radiation.

The Filter Method¹.

To get a rough estimation of the energy output in the *spectral range* 260—315 $m\mu$ of different mercury lamps the following filter-method according to KREFFT and RÖSSLER (1936) was employed (see also KREFFT, 1942 b).

The lamp was placed in the centre of an integrating Ulbricht sphere photometer (in this case a tetradecahedron, fig. 15), the inside surface of which was painted with the white UVR paint. In a small opening, screened by an aluminium plate from the direct radiation of the lamp, a filter combination was placed with the spectral transmission seen in fig. 16, and behind the filter a large selenium barrier-layer photocell, connected to a sensitive low-resistance galvanometer. The reading of the galvanometer is then a measure of the energy output of the lamp in this wavelength range, proportional to the mean spherical radiation power. The reading was compared with the same reading of the UV-Normal, where the output in this range is known in absolute units. This method applied to the LK 120 lamp gave the preliminary value of 4.8 watts for the radiation between 260 and 320 $m\mu$. Different LK 120 lamps showed very small variations.

The values obtained in this way are, however, only correct when the relative spectral energy distribution of the test lamps is identical with the UV-Normal. This is not the case with the LK 120 lamp, where the outer bulb decreases the intensities of the shorter wavelengths. If the relative intensity relations between the different wavelengths in the test lamp radiation are known, a correction

¹ The measuring equipment described below was provided by the Royal Institute of Technology, Department of Applied Electrical Engineering, Stockholm.

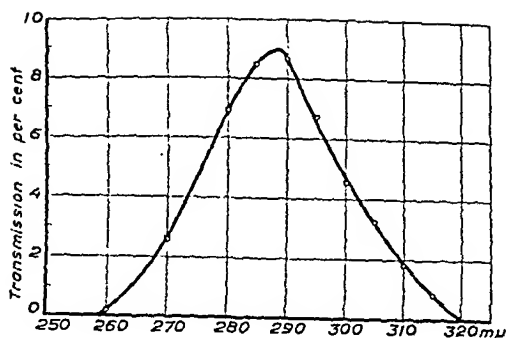


Fig. 16. The spectral transmission of the filter for isolation of the range 260—320 μ .

factor for this error can be derived. Later these relations were known for the LK 120 lamp, and the correction factor was calculated to be 0.9. The radiation flux in the wavelength range mentioned should then be 4.3 watts. This value agrees well with the sum of the values obtained for each spectral line by the photographic spectroradiometric measurements described below (see Table 2).

The composition of the filters used to select the wavelength band 260—320 μ is seen from Table 6 (KREFFT and RÖSSLER, 1936). The solution filters were kept in quartz cuvettes. The spectral transmission of the filter combination actually available for the range 260—320 μ was controlled in the usual photographic spectroradiometric way (see p. 90), with the result given in fig. 16. The transmission curve obtained is in good agreement with the original curve given by KREFFT and RÖSSLER.

The filter combination has also some transmission in the red and infrared, which is easily shown by measuring an incandescent lamp. This source of error in the measurement of ultraviolet radiation can be avoided by making a supplementary measurement with a common glass plate combined with the filters. The difference of the readings with and without this glass plate, corrected for the loss due to reflection, gives a measure of the ultraviolet radiation (method of differential measurement). For mercury lamps this correction may, however, be neglected.

The measuring equipment available included two other filter combinations, intended to select the ranges 320—355 μ and 350—410 μ , with the compositions given in Table 6. In the spectroradiometric control of the transmission limits given for these filters it was found, however, that the filter for the band 320—355 μ also had a considerable transmission in the short-wave range 260—270 μ , which limits its usefulness. The *long-wave UV-filter combination*, where the transmission limits were checked to be 350—400 μ , has been used for measurements of the strong mercury line at 366 μ . As compared with the UV-Normal, the energy flux of this wavelength from the LK 120 lamp was 3.9 watts. This is the value given in Table 2.

This filter method has proved valuable for making rapid estimations of the approximate energy distribution of different radiations and to look for variations between different specimens of the same lamp. A serious, but inevitable disadvantage is that it does not give any information about the relative energy distribution within the important range 260—320 μ . A displacement of the relative wave-

Table 6. *Composition of Filters for Isolating Different Spectral Regions of Ultraviolet Radiation.* (From KREFFT and RÖSSLER, 1936.)

260-320 mμ	320-355 mμ	360-410 mμ
SCHOTT filter UG 5	SCHOTT filter UG 5	SCHOTT filter UG 2
BÄCKSTRÖM filter	BÄCKSTRÖM filter	„ „ GG 2
Picric acid (10 mm, 0.063 %)	Nitric acid (10 mm, 0.3 N)	Cupric sulphate (10 mm, 5.7 %)

The BÄCKSTRÖM filter: $\left\{ \begin{array}{l} \text{Ni SO}_4 \cdot 7 \text{ H}_2\text{O} \quad 49.2 \% \\ \text{Co SO}_4 \cdot 7 \text{ H}_2\text{O} \quad 14.1 \% \end{array} \right\}$ thickness 20 mm.

length intensities in this range may change the relation of the erythema- to the ophthalmia-efficiency and probably also influence the yield of vitamin D. These factors made necessary more accurate measurements of the ultraviolet spectrum.

The equipment was easily adapted for measurements of the *luminous powers* of different lamps. The filters and the photocell were exchanged for a Weston Photronic cell, with the spectral sensitivity curve corrected by filters in accordance with the normal luminosity curve of the eye. The readings were calibrated in lumens after standardization against an incandescent normal-lamp.

The Spectroradiometric Method¹.

The more accurate determinations of the spectral energy distribution below 350 mμ, in the radiation from different UV-sources, also in absolute units, have been made on the principle of *heterochromatic photometry* (see, e. g., SAWYER, 1945), with the UV-Normal as the reference standard source.

Procedure. Series of spectrograms of the horizontally emitted radiation from the test lamp and the standard lamp, for the same distance, were taken successively with a quartz spectrograph (FUESS, Berlin) on spectral process plates (Ilford, or Agfa Autolith), with the exposure time increasing in logarithmic steps. The optical densities ($= \log 1/d$, d = transmission factor) of all the spectral lines in every spectrogram were then measured with the aid of a special ZEISS microphotometer. For each line the density curve was plotted on semi-logarithmic paper, with the exposure time on the logarithmic abscissa and the density on the ordinate. In such a diagram the curves be-

¹ For these measurements instruments were made available by the Royal Institute of Technology, Department of Physics, Stockholm.

come straight lines over a wide exposure range, the gradient varying linearly with the spectral contrast factor of the plate emulsion. The mutual relation between the intensity of a line in the test lamp and the normal lamp radiation is then obtained as the reciprocal of the exposure times which give the same density on the straight part of the curve.

To avoid any influence of the development of the plates, the density curves compared were always taken from the same plate or from plates handled simultaneously. The galvanometer scale of the microphotometer was calibrated to give the densities at once and the readings were directly plotted on the semi-logarithmic diagrams.

As the spectral intensities of the UV-Normal radiation are given in absolute units, the ratios obtained for the test-lamp radiation could be transformed to absolute units, the accuracy mainly depending on the reliability of the standard lamp.

Evaluation of spectral radiant flux. The data obtained in this way give the density of radiant energy for each spectral line in the horizontally emitted radiation. To get from these data the total energy (flux) of each wavelength emitted by the source the *intensity distribution curve* must be considered. This was determined, as the average of radiation of wavelengths below 315 m μ , by means of the filter-difference method described below. The result in the case of the LK 120 lamp is plotted in the polar diagram of fig. 9 (p. 67). The intensities (I) at different angles, being the averages of several meridians, are expressed as percentages of the horizontal intensity. The flux (F) between the angles θ_1 and θ_2 is then obtained from the following formula (cf. BOAST, 1942):

$$F_{\theta_1-\theta_2} = 2\pi \int_{\theta_1}^{\theta_2} I \cdot \sin \theta \cdot d\theta$$

or, if the mean value of the intensity between the limits θ_1 and θ_2 is taken as a constant of the integration,

$$F_{\theta_1-\theta_2} = 2\pi I_{mean} \left| (\cos \theta_1 - \cos \theta_2) \right|_{\theta_1}^{\theta_2}$$

Every 10 degree zone from 0° to 180° was treated in this way, the sum giving a relative measure of the total flux in the spectral region below 315 m μ emitted by the source. As the horizontally emitted radiation is known in absolute units for each wavelength, the total flux of each wavelength can also be expressed in absolute units. It has been assumed that the distribution curve is similar for all the wavelengths below 315 m μ .

In this way the values given in Table 2 were obtained.

This method of getting the spectral energy radiation of a source is rather laborious, as the density curves of about 30 spectral lines in

the range below 350 m μ have to be obtained, each of which from at least five density readings. Obviously the method is not suitable for routine measurements. Because of that, and as these measurements are of such fundamental importance in testing sources for UV-illumination, the following simple but approximate method may be recommended.

A Semi-quantitative Spectrographic Method.

This method, which has been developed during the present investigation, is based on the principle of varying the exposure on the photographic plate of a spectrograph, in such a manner that the threshold of macroscopic blackening for different intensities is reached at different heights of the plate. This aim has been realized by moving the plate past the exit slit of the spectrograph with a velocity, varying during the time of exposure according to an exponential function. The same result may also be obtained by using a logarithmically cut sector rotating before the entrance slit, as described in standard handbooks, (see, e. g., LEWIS, 1946; SAWYER, 1945), but then the quantitative spectrogram on the plate is not higher than the length of the slit. By the plate-moving method the total height of the plate can be utilized.

The *theory of the method* is as follows. From the reciprocity law of the photographic plate emulsion it is assumed that the product of radiant intensity (E) and exposure time (t) is a constant for the threshold blackening,

$$(1) \quad E \cdot t = c_1$$

The spectral plate is moved upwards, past the exit slit of height a , with the decreasing velocity $dz/d\tau$, where z is the scale of the plate height, measured from the initial stationary spectrogram, and τ the time for the displacement. At the end of the displacement the plate has moved the distance $z = k$, i. e., k is the height of the utilized part of the spectral plate (see fig. 17).

The velocity should vary in such a way that the threshold exposure $E \cdot t = c_1$ should be exceeded at a point where the distance ($k - z$) is directly proportional to the intensity E , thus,

$$(2) \quad (k - z) \cdot t = c_2$$

If the exposure time t , at $z = a$, is taken as unity, it follows that $k - a = c_2$, or generally

$$(3) \quad E \cdot t = k - a$$

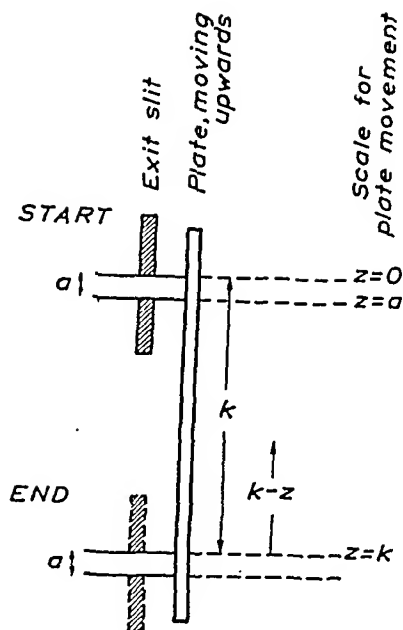


Fig. 17. Schematic representation of the semi-quantitative spectrographic method. The upward movement of the plate may be imagined as a downward movement of the slit. See text.

The exposure time t is at every point dependent on the velocity of the plate past the slit according to

$$(4) \quad t = \frac{a}{dz/d\tau}$$

By substituting $c_2 = k - a$, and the value of t from above, in eq. (2), it follows that

$$(5) \quad \frac{a \cdot d\tau}{dz} = \frac{k-a}{k-z}$$

from which z is obtained:

$$(6) \quad z = k(1 - e^{-\frac{a}{a-k}\tau})$$

The differential quotient $dz/d\tau$ of this equation is the velocity at which the plate should move past the exit slit.

The *practical arrangement* is seen in fig. 18. The curve of eq. (6) is solved numerically between the limits $\tau = 0$ and $\tau = 200$ seconds, with $k = 70$ mm (utilized height of the plate) and $a = 2$ mm (height of exit slit). This curve has been cut in a metal plate in the form of a slot, into which a small wheel is placed. The two ends of the axis of the wheel are attached by two arms to the plate-holder of the spectrograph. The metal plate with the slotted curve is moved by a small motor with a constant velocity v above the plate-holder,

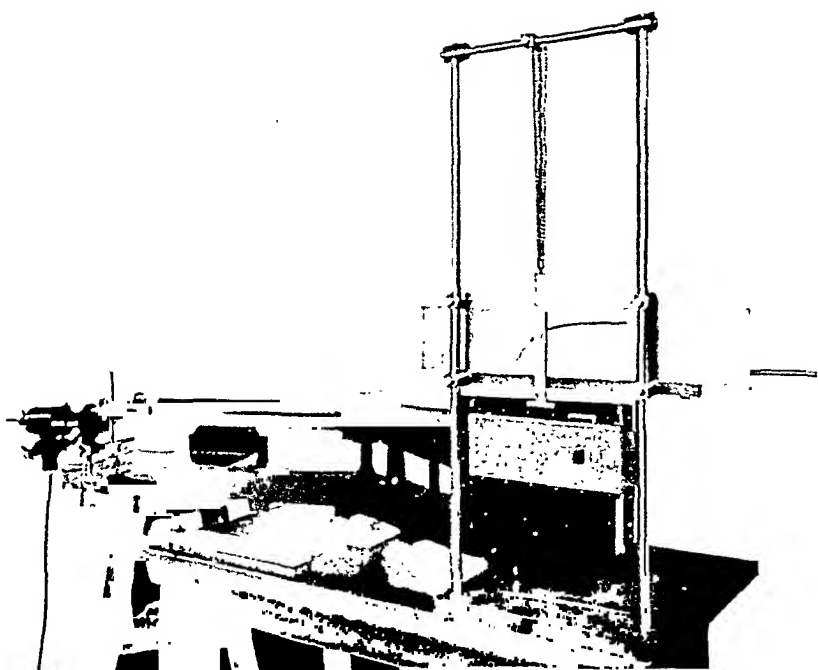


Fig. 18. The spectrograph equipped with the arrangement for semi-quantitative spectrography. A rotating sector is placed in front of the spectrograph.

and the wheel with the attached plate-holder runs up along the curve, pulled by a spring (see fig. 18). The time τ in the equations above is then given by the ratio L/v , where L is the length of the horizontal projection of the metal curve, and v is the adjustable speed of the movement of the metal plate.

The intensity of radiation on the entrance slit suitable for the procedure may be varied by the distance of the source, or by a rotating sector placed in front of the spectrograph. A pre-exposure is first made, with the plate at the lowest position, giving a narrow spectrogram at the upper part of the plate. Then the motor is started, and the plate moves upwards. At the end of the displacement the exposure is continued until a distinct after-spectrogram is obtained at the bottom of the plate.

From the developed plate a print is first taken on a hard lantern slide or photographic paper, and then a new print is made from the first one. *A line spectrum is then seen as black lines of different heights, corresponding to the different intensities.* The heights should be read from the upper end of the after-spectrogram. To get a very distinct upper limit of the lines on the prints, it was found suitable

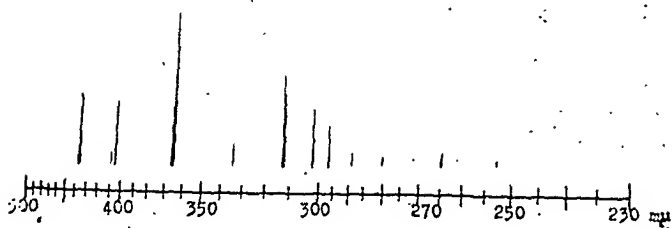


Fig. 19. A semi-quantitative spectrogram of the LK 120 lamp radiation.

to use a wet printing procedure from the first paper print to the second paper. In this way the spectrograms of figs 19—21 were obtained.

The first limitation of the accuracy of the method is obviously that the reciprocity constant (c_1) is not constant over the entire spectrum. This makes comparisons between different wavelength intensities on the same spectrogram approximate. For ordinary spectral process plates the sensitivity, however, is rather constant in the wavelength range 250—400 μ , i. e., the region of interest for most biological purposes (see MEES, 1931; and for Autolith plates see SEITZ and MEYER, 1942). This error may be avoided by comparison for each wavelength with a standard lamp spectrum obtained in a similar way.

For a given band of wavelengths, the energy content is approximately proportional to the *area* of the blackening. As the wavelength scale in prism spectrographs is spread out towards shorter wavelengths, the actual *height* of the blackening in such semi-quantitative spectrograms becomes smaller for the same spectral energy content at shorter wavelengths. Accurate readings of the semi-quantitative spectrograms must then be made by means of *area measurements* instead of height measurements. The area measurement can be conveniently carried out by reflection or transmission determinations of the selected parts of the paper prints.

The photographic treatment of the plates, especially the steps in the reproductions, involve further errors in the method. It has been

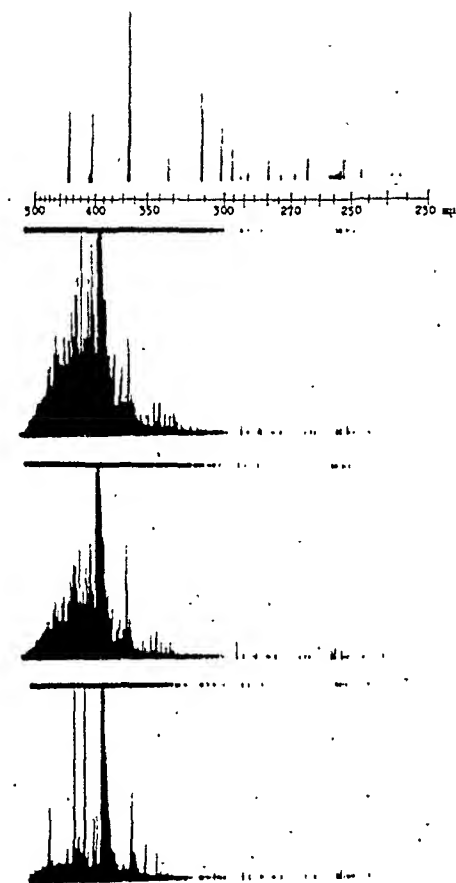


Fig. 20. Semi-quantitative spectrograms of the UV-Normal lamp radiation (uppermost spectrogram) and different types of carbon arcs (Spectram, Stockholm).

found, from series of measurements of different radiant sources, that the error of the method with careful handling is of the order of magnitude of ± 25 per cent. For most biological purposes such an accuracy is sufficient.

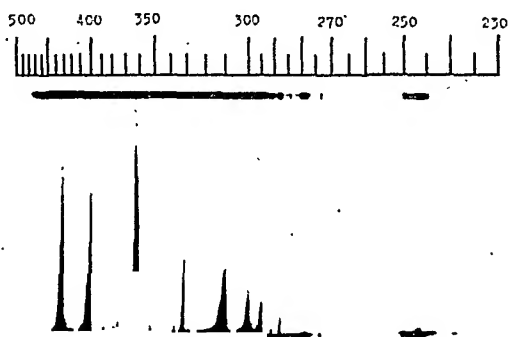


Fig. 21. Semi-quantitative spectrogram of a high-pressure quartz mercury arc (HBO 500, Osram).

Measurements of Spectral Transmission and Reflection¹

The determinations of the ultraviolet absorption or transmission curves of different filters and glasses used in the investigation have been made in the usual spectroradiometric way, by means of a ZEISS double-sector spectrometer (Spectrograph Qu 24), with a HÜFNER quartz prism as the beam-splitting device. A full description of the method employed is found, e. g., in HEILMEYER (1943).

The same arrangement was also used for the spectral reflection measurements of painted and otherwise treated surfaces. A circular

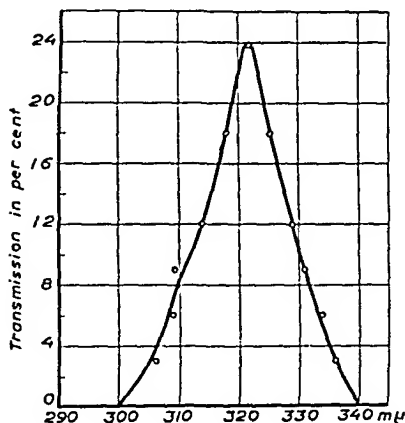


Fig. 22. Spectral transmission of the silvered quartz plate used for ultraviolet photography.

plate covered with evaporated magnesium oxide was attached to the centre of the test plate, and used as standard for the measurements. This combination was rotated in front of the spectrograph and illuminated from the side with a mercury arc quartz lamp. The standard MgO-plate was projected into the slit of the prism to form one beam, the intensity of which was varied with the adjustable sector. The beam reflected from the test plate was reduced to a constant intensity by the other part of the

double-sector. The density comparisons were estimated by the eye.

A striking illustration of the difference in ultraviolet-reflecting power of different surfaces was rapidly obtained by *ultraviolet photography*, using a mercury arc quartz lamp as the light-source (see fig. 12). The glass lens of the camera was exchanged for a quartz lens, but a stenopaic hole instead of the lens was also satisfactory for this purpose. A quartz plate, covered with a thin layer of silver according to the method of BRASHEAR (see STRONG, 1944), in combination with a SCHOTT UG 5 filter, was placed in front of the camera. The spectral transmission of the silvered quartz plate is shown in fig. 22 (cf. VON ANGERER, 1944).

¹ Most of these measurements were carried out at the Military Research Institute, Department I, Stockholm. The reflection measurements were made in collaboration with Dr O. QUENSEL at this Institute.

Measurements of Ultraviolet Radiant Intensities.

The determinations of actual surface intensities of visible and ultraviolet radiation have been made with *selenium barrier-layer photocells*, covered with suitable filters, in connection with a low-resistance galvanometer (mainly the Multiflex). The readings obtained by such a device are functions of the spectral energy distribution of the radiation, the spectral transmission of the filter and the spectral sensitivity of the photocell. With suitable filters it is sometimes possible to imitate the spectral sensitivity curve of a special photochemical or photobiological process, as for instance the luminosity curve is imitated in colour-corrected illumination meters. The readings are then directly obtained in weighted values. Otherwise, adequate comparisons between different readings are only allowable when the spectral energy distributions of the compared radiations are identical, or are known in relative values for both radiations (see KREFFT, 1942 b). Another limitation of these photoelectric measurements is that the photocell response does not correctly follow the cosine law for oblique incidence (see LARCHÉ, 1942). In common illumination measurements allowance for this error is, however, usually not made.

Unfortunately, there are no glass filters available for the selective isolation of ultraviolet radiation below about 315 m μ . Special phototubes exist which have their long-wave sensitivity limit in this region, making them suitable devices for such measurements (cf. p. 20). There is, however, a possibility to make use of selenium cells for these measurements also, namely by the *filter-difference method*. A survey of the applicability of this principle is given by SEITZ and MEYER (1942, 1943) and HENSCKE and SCHULZE (1942). In this investigation the following simple method was used.

For every intensity determination a reading was first taken with the exposed photocell covered by a thin quartz plate, and then another reading with the photocell covered by an ordinary glass plate. The difference of these readings measures the radiation which is transmitted by the quartz plate but not the glass plate (see fig. 23). The quartz plate is used in order to give equal reflection conditions. The long-wave limit of the device used was at about 355 m μ , as found by the transmission measurements of the plates. In measuring mercury radiation, where the range below this limit only contains the weak line at 334 m μ before the strong lines about 300 m μ and shorter, *the response of the device becomes mainly a measure of the biologically important range around and below 300 m μ* . Ordinary incandescent light does not give any perceptible difference-reading.

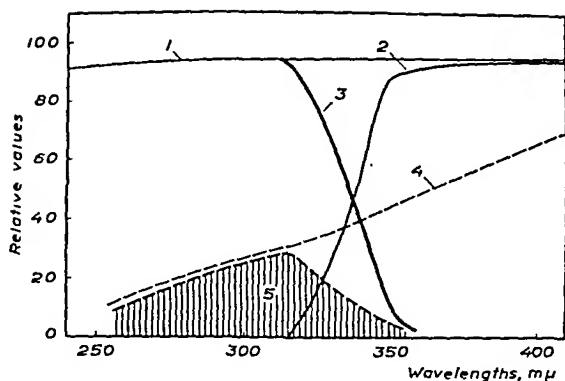


Fig. 23. The principle of the filter-difference method. 1 = transmission of the quartz plate; 2 = transmission of the glass plate; 3 = difference of 1 and 2; 4 = spectral sensitivity of the photocell; 5 = resultant spectral sensitivity of the filter-difference equipment.

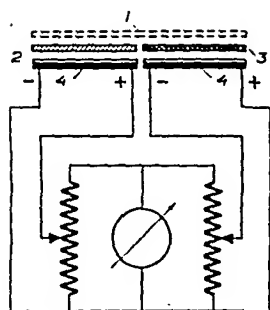


Fig. 24. Circuit arrangement of the balanced photocells for direct filter-difference readings. 1 = glass plate for zero adjustment; 2 = quartz plate; 3 = glass plate; 4 = photocell.

Instead of making the two readings successively it is possible to balance such a quartz-covered photocell against a glass-covered one and to *measure the difference directly* (see MÜLLER, GARMAN, and DROZ, 1946). As it was found difficult to find two photocells sufficiently equal for this purpose, a large selenium cell was divided into two exactly equal halves, connected according to the wiring diagram of fig. 24. The zero adjustment of the device was made with a thick glass plate covering both halves of the selenium cell.

By such devices the determinations of the ultraviolet distribution curves of figs 9 and 11 and the measurements of the ultraviolet intensities prevalent in the experimental installations have been carried out.

CHAPTER 8.

A Comparison with Daylight Ultraviolet Conditions.

The purpose of the studies reported here was to find out the approximate relations between the artificial radiation climate obtained with the UV-illumination system and the natural outdoor radiation conditions in Scandinavia, with *special reference to antirachitic ultraviolet radiation*. The studies carried out are purely theoretical and do not intend to give more than a general picture of the subject.

Experimental investigations of the radiation climate of the Scandinavian Peninsula are reported by AURÉN (1939, 1942), but these studies do not include the short-wave part of the ultraviolet spectrum.

From the great number of investigations of the daylight content of antirachitic ultraviolet radiation, i. e., radiation of wavelengths shorter than 313 m μ , (for literature see BÜTTNER, 1938; IVES and GILL, 1937), the first general conclusion is that this short-wave spectral component varies to a much greater extent than the visible and infra-red radiation by *changes of the sun elevation* and also the *altitude above sea-level*. The short-wave limit is spectrographically found to be from 290 to 315 m μ , depending on the sun elevation (see BÜTTNER, 1938). In an extensive investigation TISDALL and BROWN (1931) showed that when rachitic rats are exposed for two hours to noon daylight there is a marked antirachitic effect only when the sun is more than 35 degrees above the horizon. MAYERSON and LAURENS (1930, see also LAURENS and MAYERSON, 1933) have given further evidence of the marked seasonal variation of the antirachitic effect of sunshine.

Another general conclusion of importance is that for medium and low elevations of the sun the *diffuse sky radiation* contributes considerably *more* than the direct sun radiation to the intensity of this spectral component at the surface of the earth. This has already been pointed out in the pioneer work of DORNO (1919). The biological importance of sky radiation is experimentally proved by MAI (1938, 1939).

The general validity and applicability of the different investigations carried out are, however, in many cases limited, because the indicating or integrating instruments employed have not been sufficiently well-defined with respect to the spectral sensitivity and to the correct response to sky radiation. As the short-wave limit of the sun's intensity spectrum is very abrupt, the intensity at 340 m μ for 40° sun elevation being about 100 times that at 300 m μ and 2 000 times that at 295 m μ , the *long-wave spectral sensitivity limit* of the measuring device is of decisive importance for the results obtained. Considerable sensitivity above 313 m μ is, for instance, a disadvantage of the UV-Dosimeter of I. G. FARBEN which has been widely used in Europe, and with which investigations have been made for many different localities and latitudes (see BÜTTNER, 1938; LANDSBERG, 1937; for northern latitudes SYDOW, RIEMERSCHMID, and TIEDEMANN, 1939).

It seems, however, possible to obtain a general survey of the geographical and seasonal variations of the short-wave ultraviolet, without referring to any special measuring device, from the measurements of the actual spectral energy distribution, in absolute units, of the extreme ultraviolet of the solar spectrum, recently carried out at the *Bureau of Standards, Washington*, by COBLENTZ and STAIR (1936 a-b). The principle of their extensive investigations has been to calculate, for different elevations of the sun, the exact energy distribution below and including 313.2 m μ from a set of simultaneous measurements with photoelectric and thermoelectric devices of somewhat different spectral sensitivity within this wavelength range. *The following calculations are mainly founded upon the average data of these papers.* It is assumed that a given air mass causes the same depletion of the radiation at the Scandinavian latitudes as at the Washington latitude. The calculations refer only to sea-level.

O'BRIEN (1943) has published calculations of the annual and daily variations of the antirachitic, erythema and bactericidal efficiency of solar radiation, in relative values, for the latitudes 33, 38 and 43 degrees, using approximately the same principles of calculation as have been applied here. His calculations refer only to the intensity variations, while the calculations reported here also include the *dosage* variations. To the extent to which they are comparable the agreement seems satisfactory.

The Relation of UVE-Intensity to the Sun's Elevation.

From the tables and diagrams given in the papers of COBLENTZ and STAIR the diagram in fig. 25 has been calculated and plotted, showing how the average intensities of different wavelengths below 315 m μ in the solar radiation vary with the mass of air traversed, i. e., the elevation of the sun. The fact that the plot of the decrease of intensity in the semi-logarithmic diagram is a straight line shows that the absorption in the atmosphere follows in the main an exponential law. The thickness of the mass of air traversed by the rays (m) is found from the sun's elevation (h) by the formula

$$m = \frac{1}{\sin h}$$

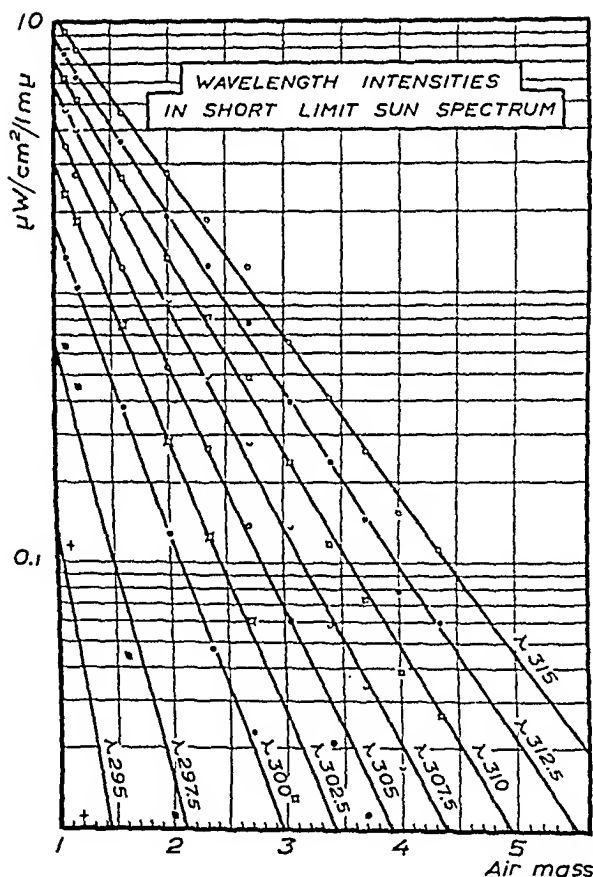


Fig. 25. The intensity depletion of different wavelengths in the short-wave end of the solar spectrum with increasing ray length through the atmosphere (air mass). The unit air mass is the length of the path through the atmosphere if the elevation of the sun is 90° (cf. fig. 26). The diagram is computed and plotted from data given by COBLENTZ and STAIR (1936 a-b).

i. e., the mass of air is measured in no. of atmospheres, with the zenith atmosphere ($h = 90^\circ$) as unity (cf., e. g., CONRAD, 1942). The general mathematical expression of the curves in the diagram is then

$$I_{m\lambda} = I_{0\lambda} \cdot (q_\lambda)^m$$

where $I_{m\lambda}$ = the intensity of wavelength λ for mass of air = m ,
 $I_{0\lambda}$ = the corresponding extra-terrestrial intensity ($m = 0$),
 q_λ = the transmission coefficient $\left(\frac{I_{1\lambda}}{I_{0\lambda}}\right)$ for the wavelength λ .

The curves given are valid for the intensities perpendicular to the direction of the sun's radiation and only for sea-level. Sky radiation is not included.

Table 7. *Wavelength Coefficients for the Evaluation of UVE-Radiation.*

Wavelength m μ	Erythema factor I.C.I.	Antirachitic factor		UVE- coefficient adopted
		KNUDSEN- BENFORD	BUNKER- HARRIS	
295	0.98			1.0
297.5	1.0	1.0	1.0	1.0
300	0.83			0.8
302.5	0.55	0.48	0.47	0.5
305	0.33			0.3
307.5	0.20			0.2
310	0.11			0.1
312.5	0.03	0.01	0.0	0.02
315	0.01			0.00

Every ordinate in this diagram includes values of the average spectral energy distribution, in absolute intensity units, for the corresponding air mass, i. e., sun elevation, and may be used as a nomogram for the equation given above. By weighting the *energy distribution curves* plotted from this diagram against the *action spectrum* of a photobiological response, a series of *product curves* of weighted spectral intensities is obtained for the different sun elevations. The surface integrals of the product curves give the total intensity of weighted incident energy, expressed as the equivalent intensity of the reference wavelength in the action spectrum (see fig. 3, p. 47).

From the point of view of human physiology the *antirachitic effect* and the *erythema effect* are of special interest. The relative shapes of the action spectra for these two effects happen to coincide almost exactly for wavelengths longer than 295 m μ , i. e., the short-wave limit of the solar spectrum, if the action factor for the wavelength 297 m μ is taken as unity for both these curves (see fig. 4, p. 51). For the purpose of the following calculations an *average action spectrum* was adopted for the biologically-effective radiation (UVE) in the solar radiation. The wavelength coefficients selected are given in Table 7. Wavelengths longer than 315 m μ have not been given any weight, as they do not possess any antirachitic properties. They have, however, a slight erythema effect, or rather a tanning effect, on human skin, which may contribute about as much to the total tanning effect of solar radiation as the wavelengths below 315 m μ (see p. 51). *The UVE-values obtained by the action spectrum adopted here*

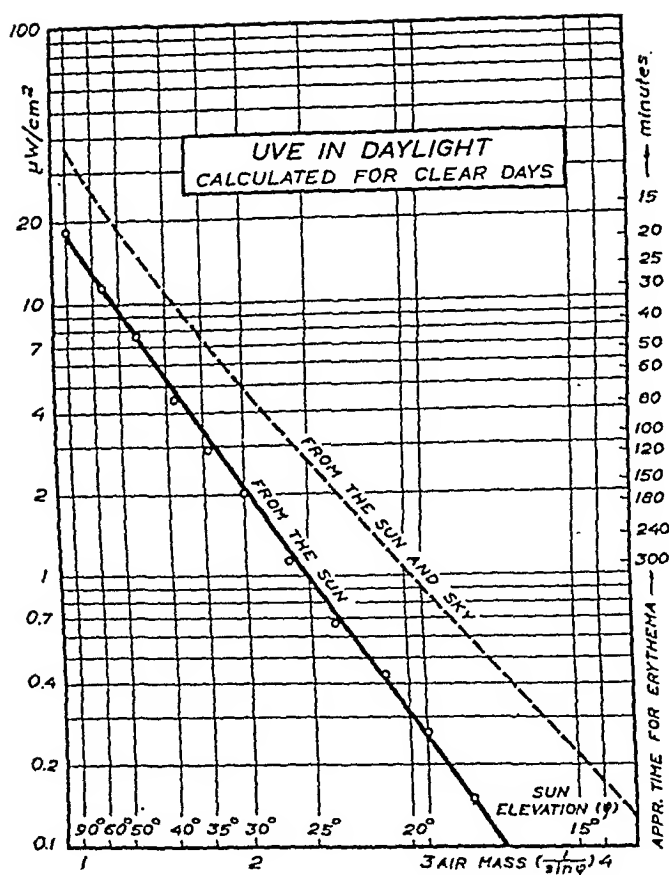


Fig. 26. Calculated intensities of erythemally-weighted ultraviolet radiation of wavelengths below 315 m μ (UVE) in solar radiation (perpendicular to the rays) and of sun and sky radiation on a horizontal surface at different elevations of the sun.

will then be somewhat too small with regard to the skin-tanning property of the radiation, but will fit the antirachitic efficiency closely.

Fig. 26 shows the UVE-intensities obtained in this way as a function of the mass of air. As expected, it also gives a straight line when plotted on a semi-logarithmic diagram. The unbroken curve is valid for the solar radiation, perpendicular to the direction of the rays. It corresponds to an effective transmission coefficient (q) of about 0.10.

The relative contribution of sky radiation to the short-wave ultraviolet intensity at the earth has been measured by, among others, BÜTTNER (1938) and LUCKIESH, TAYLOR, and KERR (1939). In spite of the different methods employed, the results are in almost perfect agreement when expressed in a similar way. The ratio of sky radiation to sun radiation on a horizontal surface equals unity at sun elevations

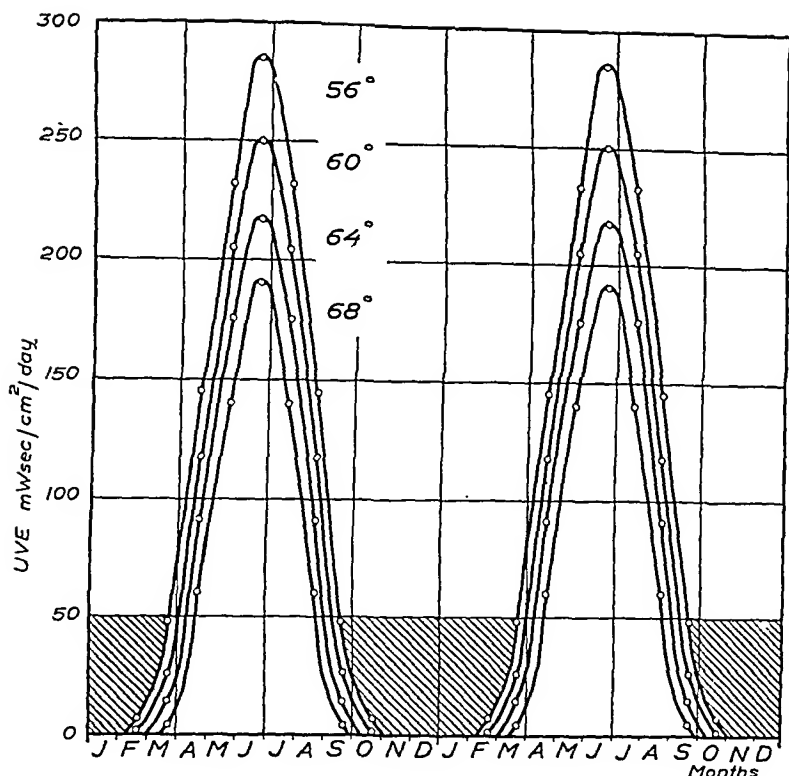


Fig. 27. Calculated daily amounts of UVE arriving at a plane perpendicular to the solar radiation for different seasons of the year and at different Scandinavian latitudes. The shaded level denotes the amount (dosage) required for producing a slight degree of tan on the average unpigmented human skin. The corresponding curves for sun and sky UVE incident on a horizontal plane are practically identical if the ordinate scale is doubled.

above 70 degrees, provided that the air is clean, and rises to about 10 at a sun elevation of 20 degrees. By using the mean quotient obtainable from these investigations for every sun elevation, the UVE-intensity on a *horizontal surface from both sun and sky radiation* was calculated, with allowance of course for the cosine law for the direct sun radiation. The result is shown by the broken curve of fig. 26.

It is seen from this diagram that the UVE-intensity $1.5 \mu\text{W}/\text{cm}^2$ obtained by the artificial UV-illumination system in the experimental schoolrooms corresponds to the UVE-intensity of direct solar radiation for a sun elevation of about 30 degrees above the horizon, or to the intensity falling upon a fully exposed horizontal surface on a clear day for the sun elevation 20—25 degrees. It may be mentioned, that the midsummer sun elevation in the south of Sweden reaches a maximum of about 55 degrees, and in the north of Sweden about 45 degrees.

The corresponding exposure times calculated to produce a threshold erythema for the different UVE-intensities are given by the ordinate on the right of fig. 26. The threshold dosage was presumed to be 20 mWsec/cm². The data are in good agreement with similar time values given by BÜTTNER (1938) for the exposure necessary to produce a slight tan of normal skin for different sun elevations. The intensity values of the diagram agree well with some corresponding data also given by LUCKIESH and TAYLOR (1945).

Daily Dosage of UVE at Different Seasons and Latitudes.

From the photobiological point of view the intensities of UVE-radiation in daylight are of less interest than the *daily dosages* available for different seasons and latitudes. Actual measurements of the daily amount of tanning-active ultraviolet radiation have recently been made during a six year continuous record by LUCKIESH, TAYLOR, and KERR (1937, 1939, 1944; see also LUCKIESH, 1946), at a latitude of 41.5° N. The calculations made in the present investigation mainly refer to Scandinavian latitudes, but for the comparison they have also been made for lower latitudes.

With the aid of the diagram in fig. 26 and of the curves available for the apparent course of the sun across the sky on different days of the year and in different latitudes, the *daytime variation curve* of the UVE-intensity was plotted in diagrams for six pairs of corresponding days in the year. *The surface integral of such a curve gives the total UVE-energy (UVE-dosage) incident for that cloudless day.* Fig. 27 summarizes these integrated data obtained for different seasons and different Scandinavian latitudes, with respect to the *sun radiation* perpendicular to the rays (sky radiation not included). Similar day-dosage curves were also calculated for *sun and sky radiation* on a horizontal plane, and it was found that the course of these latter curves was in almost perfect agreement with the curves in fig. 27 if only the ordinate scale were doubled.

It is seen from the figure that there is *practically no antirachitic radiation (UVE) at all reaching the earth's surface during 4—6 months of the year, at the Scandinavian latitudes.* With the UV-illumination the daily exposure was maintained during the winter at a level of about 20 mWsec/cm² of UVE-radiation.

Annual Dosage of UVE at Different Latitudes.

The surface integrals of the day-dosage curves of the year (fig. 27) permit a computation of the theoretical value of the *total UVE-energy maximally available* during a presumed cloudless year. Such values, including *UVE-radiation from sun and sky reaching a horizontal surface* are calculated for different latitudes, with the result shown in fig. 28. The broken line indicates the total heat insolation, in relative values, for a horizontal surface, given as annual averages for clear days (see CONRAD, 1942). *The much greater influence of the geographical latitude on the amounts of ultraviolet radiation reaching the earth as compared with the total heat radiation is evident from the figure.*

The total annual UVE-radiation calculated in this way for the latitude of 40° is, as seen from fig. 28, $125,000 \text{ mWsec/cm}^2$. This value should be compared with the average value actually found in the integrating photoelectric measurements of LUCKIESH, TAYLOR, and KERR, mentioned above, which was 3,000 E-viton hours, or $110,000 \text{ mWsec/cm}^2$ of tanning-effective radiation. Allowing for the mean cloudiness and the fact that their measuring device was also somewhat sensitive for wavelengths longer than $315 \text{ m}\mu$, the agreement is surprisingly good.

The calculations reported here are obviously only of a general nature, giving an estimation of the theoretical maximum values of UVE-energy available at different latitudes and different seasons. *The real ultraviolet radiation climate of a locality* is dependent upon many other factors, such as the cloudiness, the albedo of the earth's surface, the altitude, and local and seasonal variations of the transmittance of the atmosphere. The content of ozone in the atmosphere exerts a great influence on the spectral energy distribution of the short-wave ultraviolet spectrum, and this content is known to vary considerably from day to day. The effect of this is clearly demonstrated in the calculations of O'BRIEN (1943). In conclusion, however, the deciding factor from the hygienic point of view is the degree to which the outdoor radiation climate is really utilized by human beings.

The effect of cloudiness and snow. — It is found by BÜTTNER that cloudiness generally diminishes the ultraviolet radiation to a less extent than visible light and heat radiation. The spectral composition in the UVE-range is not al-

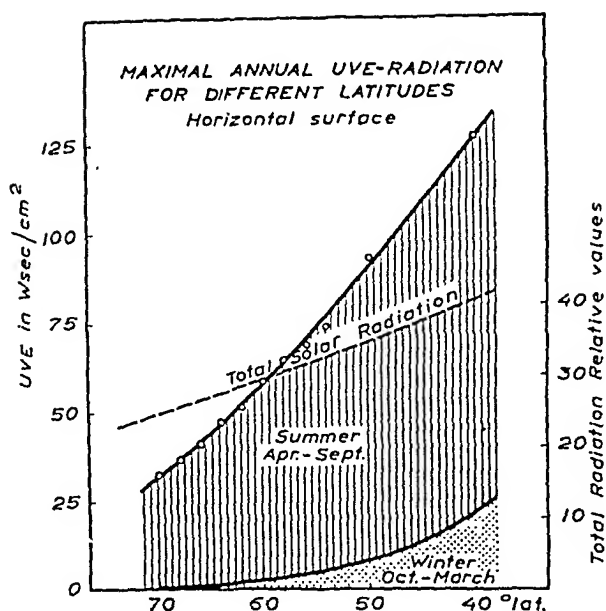


Fig. 28. Calculated amounts of UVE reaching a horizontal surface directly from the sun and entire sky for a presumed cloudless year at different latitudes. The curve of total solar radiation gives the same data (in relative units) for the total of ultraviolet, visible and infra-red radiation.

tered by clouds or fog (COBLENTZ and STAIR, 1935). When the sky is entirely covered by clouds (cloudiness 10) the UVE-intensity at the earth is diminished to about 50 per cent of the corresponding clear sky intensity, as measured with the UV-dosimeter (see BÜTTNER, 1938). The average number of hours of sunshine during the summer months in Sweden, according to ÅNGSTRÖM (1946), is 45—50 per cent of the possible number. It could then be expected that about three quarters of the calculated value of the maximal annual UVE-energy reach the earth in Sweden, i. e., about 36,000 mWsec/cm². During the winter months the average cloudiness is still greater, but on the other hand the snow-cover causes at least a doubling of the mean radiation intensity by reflection (see BÜTTNER, 1938).

An obvious consequence of the data obtained by these calculations is that the use of special *ultraviolet-transmitting glass in windows* in order to get an improvement of the indoor radiation climate is futile during the winter, at least at the latitudes of Scandinavia. The ineffectiveness of such devices for general use was pointed out long ago by CLARK (1928) and KOLLATH (1929), see p. 36. Hence the only way to attain this purpose is to make use of artificial sources of ultraviolet radiation.

Table 8. *Intensities of Visible and Ultraviolet Radiation in Daylight (Clear Days). Estimated Values.*

Component of Radiation	Sun Elevation 60°		Sun Elevation 30°	
	Direct Sun	Sky	Direct Sun.	Sky
Light	100,000 lux	17,000 lux	75,000 lux	15,000 lux
UV 400—315 mμ	4,000 μW/cm ²	?	2,500 μW/cm ²	?
UV below 315 »	55 »	60 μW/cm ²	15 »	20 μW/cm ²
UVE »	12 »	13 »	2 »	3 »
UVK »	6 »	7 »	1 »	1.5 »
UVX »	1.6 »	2 »	0.3 »	0.5 »
UVG	12 »	(10)	4 »	(4)

The Average Daylight Ultraviolet Climate.

For the comparison of the outdoor "ideal" radiation climate with the artificially created radiation climate indoors see Table 8 which is given in the same form as Table 5, p. 77. The illumination values are according to LUNELUND (1935), the data for long-wave ultraviolet are taken from the measurements of AURÉN (1939) and the data for short-wave ultraviolet from the measurements of COBLENTZ and STAIR used above. The weighted intensities given for UVK, UVX and UVG are calculated in the same manner as was applied to UVE in the calculations reported above. The action spectra for these biological effects in the long-wave ultraviolet range (315—400 mμ) are, however, in general not so well-known as for the shorter wavelengths, which makes these values in the table rather uncertain. For both UVK and UVX, i. e., the radiation injurious to the eye, the action spectra available were arbitrarily extrapolated to 315 mμ, where the long-wave limit was placed, as done previously for the UVE-radiation. The bactericidal intensities (UVG) are computed upon the basis of the action spectrum given by LUCKIESH, HOLLADAY, and TAYLOR (see LUCKIESH, 1946), which includes data as far as in the visible spectrum. In the main this curve seems to be in agreement with the findings of other authors (cf. HOLLAENDER, 1946).

A comparison between the tables indicates that the UV-illumination offers some resemblance to the natural radiation climate at the sun elevation 20—30 degrees, but only with respect to the biologically weighted intensities of short-wave ultraviolet. For the same erythema-effec-

tive intensity, the intensity of *long-wave ultraviolet radiation* is at least 500 times greater in the natural solar radiation than in the mercury radiation of the UV-illumination. One consequence of this, as pointed out before, is that the degree of skin-tanning of the children in the illuminated classrooms was not as high as would have been reached by the same UVE-dosage of natural sunshine.

It should be noted that the intensity of ophthalmia-producing radiation in natural daylight, calculated in this way, is comparatively high, also for sky radiation alone. The reason why the effect of this is not usually observed may be the same as that assumed for the UV-illumination, i. e., the protected situation of the eyes for overhead radiation. The risk of eye-irritation in the UV-illuminated rooms could then be expected to be not much higher than outdoors during a bright summer day.

III.

The UV-Illumination Effects.

CHAPTER 9.

A General Scheme of the Experiments.

The investigation of the physiological and hygienic effects of the supplementary ultraviolet radiation in the artificial illumination, obtained by means of the UV-illumination system previously described, has hitherto been carried out in a Secondary school and a Primary school in Uppsala during a period of three school terms (one and a half years). The organisation of the school classes involved in the experiments is shown in fig. 29. Every rectangle represents a group of about thirty children, corresponding to an ordinary Swedish school class. The ultraviolet irradiated groups are marked with shaded rectangles, the control groups with open rectangles. During the last term of investigation the non-irradiated Group III was divided into two halves, one of which was treated with a large dose of vitamin D, as indicated by the half-pointed rectangle (later referred to as *The Vitamin D Experiment*). The connecting lines indicate that the same children have been followed consecutively over more than one term. *A total of about 220 children have been involved in the experiments, 120 of which have been given ultraviolet irradiation for some period of time.*

Groups I and II and, after the first term, also Group III were composed of boys only, the other groups of both boys and girls. The age distribution was very homogeneous, with practically all 6—7 year-old children in the Primary school classes and 9—11 year-olds in the Secondary school classes. The social character of the groups can be regarded as representative of a random sample of Swedish Primary and Secondary school children and without significant differences between the several groups engaged. An interview revealed that only very few of them were having their diet supplemented by vitamin preparations during the winter.

The classrooms of Groups I—III were situated next to each other along a ground-floor corridor of the Vaksala Secondary School in

SECONDARY SCHOOL CHILDREN
Age about 10 years

PRIMARY SCHOOL CHILDREN
Age about 7 years.

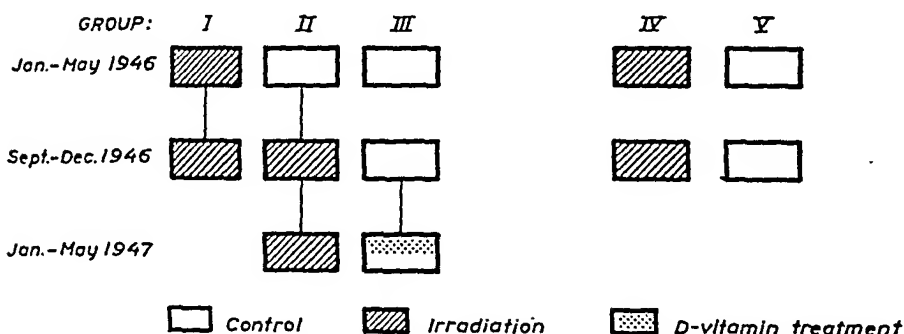


Fig. 29. The organization of the school children trial on UV-illumination. Every rectangle corresponds to a class of about 30 pupils for one school term.

Uppsala, each with four windows facing North-East and towards the playground. The opposite side of the corridor included other classrooms, not engaged in this trial. The classroom of the experimental Group IV, with Primary school children, was situated in a small private building in an outer part of the city, and no other children were taught there. The control Group V was selected from corresponding classes of the Vaksala Secondary School. The daylight illumination in all the classrooms involved in the trial was in good agreement with modern Swedish requirements (see ARNER and YLLNER, 1941).

During the first term of the investigation Group I was provided with the UV-illumination system, and in the classroom of Group II the same special fixtures were installed but without ultraviolet lamps. During the following term this room was also equipped with a complete UV-illumination system. The classroom of Group IV was provided with the complete system from the beginning. All other control rooms had common opal globe luminaires.

The investigation has included the following examinations and studies.

1. Six series of *blood analyses* on the children with regard to the following blood constituents:

- the haemoglobin concentration,
- the plasma level of calcium,
- the plasma level of inorganic phosphorus, and
- the plasma activity of alkaline phosphatase.

2. Six series of evaluation of the *physical fitness* of the children by testing with a submaximal work.

3. Determinations of the *concentration of micro-organisms in the air* in the experimental and control rooms, made by random samples during the last term of the investigation.

4. Record of the *absenteeism due to illness* among the children, supplemented with statistics of the annual course and height of the absenteeism in a number of corresponding school classes for the years 1943—47.

Records have also been made of the increase in height and weight of the children, obtained from the body measurements made in connection with the fitness tests. These measurements were, however, not made with sufficient accuracy to permit a reliable interpretation, and the material was too small to give significant differences between irradiated and control children.

The Statistical Treatments.

The statistical data of the *error of estimations* are reported in connection with the descriptions of the different methods employed. For the methods of the *blood-chemical analyses* a large number of double determinations on different specimens, and series of determinations on the same specimen, have been carried out, permitting for all methods a very exact evaluation of the random errors of the method. This error (σ_E) is calculated from the pairs of double-determinations by using the formula

$$\sigma_E = \pm \sqrt{\frac{\Sigma d^2}{2 \cdot n}}$$

where Σd^2 = the sum of the squares of the differences between the two determinations made on the same specimen, and n = the number of pairs of such double-determinations (see DAHLBERG, 1940). For the series of determinations carried out on the same specimen the error of estimation is given by the standard deviation of the series.

Special attention is also devoted to the influence of the error of the control determinations made in connection with all the series of blood analyses. The control determinations on standard solutions are used for the conversion into absolute values of the titration values or the photometer readings obtained. If M_1 = the mean reading of the series of blood determinations and M_2 = the mean reading of the series of determinations made on the known standard solution of the concentration C , the evaluated mean (M) of the series of blood determinations is obtained from the formula

$$M = \frac{M_1}{M_2} \cdot C$$

The standard error (ϵ_M) of this mean value M was calculated according to the formula

$$\epsilon_M = \pm C \cdot \frac{\sqrt{M_2^2 \epsilon_{M_1}^2 + M_1^2 \epsilon_{M_2}^2}}{M_2^2}$$

where ϵ_{M_1} and ϵ_{M_2} are the standard errors of the mean of the blood series and the standard series, respectively (see DAHLBERG, 1940, p. 95). When ϵ_{M_1} is considerably greater than ϵ_{M_2} , which was usually the case in the analyses carried out here, ϵ_M may be directly replaced by $C \cdot \epsilon_{M_1}/M_2$.

The error of estimation in the *work tests on physical fitness* was, in accordance with common use, evaluated by means of the *retest-reliability coefficient* (r_{11}), i. e., the coefficient of correlation between two administrations of the same test on a number of individuals (see GARRET, 1947). This "self-correlation" coefficient is a quantitative estimate of the importance of chance or variable influences upon the test scores owing to the method or to unaccounted day-to-day changes of the individuals. Constant errors working in only one direction do not affect the reliability coefficient. The *standard error of an obtained score* may then be obtained from the formula

$$\sigma_{1\infty} = \sigma_1 \sqrt{1 - r_{11}}$$

in which $\sigma_{1\infty}$ = the standard error of an obtained score ("standard error of measurement"), σ_1 = the standard deviation of the series of test scores, and r_{11} = the reliability coefficient of the test. This measure of the error of a single observation takes account of the self-correlation of the method as well as of the variability within the group and is the best method of expressing the reliability of a test (GARRET, p. 392). See further p. 136.

The statistical treatment of the *samples of data* in absolute values, obtained from the different kinds and series of examinations, has included the calculations of the mean value (M), the standard deviation (σ) and the standard error of the mean (ϵ) in the usual manner (see, e. g., KEMP, 1942).

In order to control that the material treated did not show apparent asymmetry of the distribution, the frequency distribution was plotted in graphs for every sample. There was in no case significant reason to make corrections for asymmetry. In doubtful cases the χ -square test was applied (see KEMP, p. 120).

When two compatible series of data of n_1 and n_2 individuals, respectively, with the mean values M_1 and M_2 , were combined to form one group, the following formulae were applied:

$$M = \frac{n_1}{n_1 + n_2} M_1 + \frac{n_2}{n_1 + n_2} M_2$$

$$\sigma = \frac{(n_1 - 1) \sigma_1^2 + (n_2 - 1) \sigma_2^2}{n_1 + n_2 - 2}$$

$$\epsilon_M = \pm \frac{\sigma}{\sqrt{n_1 + n_2}}$$

The Reproduction of the Results.

A detailed report of the very comprehensive material collected and the statistical data of it would be rather confusing. Hence the main results are given in a kind of *statistical diagrams* as shown in figs 30—38. *Every sample of data is represented by a rectangle, the height of which gives the range where the real mean value of the sample is to be found with a probability of 90 per cent.* The width of the rectangle gives an approximate measure of the number of observations in the sample (usually about .25).

The rectangles are obtained by using the *t*-factors in the following way (cf. KEMP, pp. 90—92). The standard error (ϵ) of the mean of the sample, calculated in the ordinary way, is multiplied by the *t*-factor, which corresponds to a probability of 0.1 that the real mean value would be randomly situated outside the limits $\pm t \cdot \epsilon$ around the actual mean value obtained. This means that there is a 90 per cent probability that the real mean lies within the range $M \pm t \cdot \epsilon$, which is the range indicated by the height of the rectangles in the diagrams. The *t*-factors corresponding to this probability level and to the number of the degrees of freedom in the samples were obtained from the tables of FISHER and YATES (1946). It should be pointed out that the rectangles thus give the 90 per cent probability range of the real mean of the group independent of the number of observations in it.

These diagrams do not, however, always permit an immediate evaluation of the *significance of the differences* between the means of different groups. Assuming that there is no correlation between the groups to be compared, an idea of the significance is of course given by the vertical distance between the adjacent limits of the two rectangles mutually displaced, but an exact evaluation of the significance can only be made by application of the *t*-test on differences between the means of small samples (see KEMP, p. 103). In the special cases reported here, however, the samples to be compared within the same time-series of examination (i. e., non-correlated groups) are always of about the same magnitude both with respect to the number of observations and the standard errors of the means. From this it follows that the ranges $t \cdot \epsilon$ are about equal, i. e., the rectangles $M \pm t \cdot \epsilon$ are of about the same size. If this is the case, a vertical displacement of the two rectangles to be compared, just sufficient to give a free space between them, obviously gives the relation

$$M_1 - M_2 = 2 \cdot t \cdot \varepsilon$$

The t -factor of the difference, called t_D , is in the same special case obtained from the formula

$$t_D = \frac{M_1 - M_2}{\varepsilon \sqrt{2}}$$

These two equations combined give

$$t_D = t \sqrt{2}$$

When the number of observations in each sample is about 25 (= 24 degrees of freedom), as in most cases here, the value of t is about 1.71 (rising to 1.81 for 10 degrees of freedom), i. e., $t_D \approx 2.40$ (or, for 10 degrees of freedom, $t_D \approx 2.55$). These t_D -values of the difference correspond to a probability for a difference at random of 0.05—0.01, thus indicating a "probable" difference. Moderate disparities of number etc. between the samples do not alter this general statement. This is the reason why the probability range for the rectangles in the diagrams was chosen at the 90 per cent level. *For all cases given in the present diagrams, a displacement of two rectangles representing different groups just sufficient to give a free space between them, indicates that the difference is at the limit of the usually accepted criterion of a real, significant difference.*

With increasing space between the rectangles the probability for a real difference is rapidly increasing. In fig. 33, for instance, the difference between the means of the two samples at March 1946 corresponds to a probability of much less than 0.001 for a difference at random ($t_D = 6.3$, $n = 57$).

Of course, in doubtful cases, all the possible differences between the different groups have also been tested in full accordance with the exact formula for the t -test on small samples, before any decisive conclusions have been drawn.

This interpretation of the statistical diagrams is, however, not valid for *comparisons of two means, when the underlying observations are correlated*, i. e., when a high value in one sample is also likely to be combined with a high value in the other sample. Such a correlation may be entering when two means should be compared, obtained from the same test administered to the same subjects upon different occasions, as in the study of possible *seasonal variations*. If there exists such a correlation, which, for instance, is rather high for the scores of physical fitness, the present diagrams under-estimate the significance of possible differences when read as described above. When seasonal variations have been tested for their significance in the present investigation, the mean and the standard deviation of the series of *differences* obtained for the subjects have been computed, and the mean difference then tested on the null hypothesis with the t -test. In this way the correlation is taken into account without making the calculation of the coefficient of correlation between the samples necessary (cf. GARRET, 1947).

As, however, the diagrams just described include most of the data of interest for the purpose of this investigation in a clear form and with regard also to the statistically evaluated significances of the means, it was considered unnecessary to give a complete, detailed account of the comprehensive material.

The statistical treatment reported above is of course valid only for the random variations of the samples. It is difficult completely to avoid the appearance of systematic errors in such a wide investigation of long duration. For instance, it was not possible to have the same assistants performing the same analyses in all the series, and the influence of the personal factor was sometimes demonstrated.

CHAPTER 10.

Effects on Some Constituents of Blood.

The time scheme for, and the number of experimental subjects in the six series of blood analyses carried out are seen from Table 9.

Table 9. *Scheme of Blood Analyses.*

Time	Group I		Group II		Group III		Group IV		Group V	
	Boys	Girls	Boys	Girls	Boys	Girls	Boys	Girls	Boys	Girls
Dec. 1945	27	—	25	—	9	14	6	7	—	—
March 1946	26	—	25	—	9	16	11	14	16	9
May-June 1946	25	—	24	—	9	9	11	7	11	7
Oct. 1946	29	—	31	—	29	—	13	11	14	16
Dec. 1946	25	—	30	—	29	—	12	9	13	9
March 1947	—	—	—	—	29 ¹	—	—	—	—	—

¹ 15 of whom had received vitamin D.

The reason why the last series (March 1947) did not consist of all the groups was, that the children of Groups I and II were unwilling to be subjected to further venous punctures, and for Group IV the ultraviolet lamps had become inactive and new lamps were not available. The only group examined by blood analyses during the last spring term of the investigation was therefore the class partly treated with vitamin D (Group III).

The first series (Dec. 1945) did not include phosphatase analyses, and haemoglobin determinations were carried out only in Groups II and III. The reason was methodical in origin.

The Analysis Methods Employed.

The sampling of the blood.¹ The sampling of the blood was always performed in the morning when the children had just arrived at the school. Venous puncture was made with a dry sterilized syringe, and about 10 ml of blood were allowed to flow into a thoroughly cleaned and acid-washed test tube, containing 0.2 ml of a 0.5 per cent solution of sodium heparin and some glass pebbles. The samples were centrifuged within one hour after taking the blood, and after the sample for the haemoglobin determination had been withdrawn. Analyses were carried out on 15—25 samples daily during the examination periods.

According to WILANDER (1938) there is no difference except the dilution effect in the results of blood analyses on calcium and phosphatase when using heparin plasma instead of serum. This has been confirmed, also with respect to inorganic phosphorus, by experimental controls made in the present investigation.

The Haemoglobin Method. The alkaline haematin method according to WU (see PETERS and VAN SLYKE, 1931) was employed in the following manner. After thoroughly mixing the sample, 0.05 ml of the heparinized blood was collected in 5 ml of N/10 HCl, stoppered and mixed, and left at room temperature for at least 3 hours. 0.5 ml of 10-N NaOH was then added and the solution was measured against distilled water as a blank in the ZEISS visual "Stuphen-Photometer" (Pulfrich) with 1 cm cuvette and green light filter (S 50).

The standardization of the readings was made by comparisons with the carefully calibrated Authenrieth Haemoglobinometer of Dr H. ENGHOFF at this Institute, which is used as standard for a great number of Swedish haemoglobinometers (see ENGHOFF, 1937). Standardizations were made for three of the six series of analyses, and the calibration factor obtained was in all cases exactly the same.

The values are expressed in grams of haemoglobin per 100 ml of blood ("g %") No allowance has been made for the dilution due to the heparin added.

The *error of the method*, computed as the standard deviation of 20 determinations of the same blood specimen, was ± 0.17 g %, or 1.03 per cent of the mean.

The Calcium Method. The method of KRAMER and TISDALL was employed with the procedures in accordance with the description given by KING (1946). Some other methods, which are said to be more exact, were tried but found too tedious to combine with the other analyses to be carried out on the blood samples.

The N/100 potassium permanganate solution for the titration, which was diluted from the N/10 standard solution every day of the test periods, was checked in the usual manner by titration with a standard oxalic acid solution. Further, every daily series of the plasma calcium estimations were combined with estimations of a number of specimens of an exactly weighed standard

¹ This responsible task was kindly carried out by Nurse KARIN NORDSJÖ.

calcium solution of about 10 mg %, which were handled simultaneously and in the same manner as the plasma specimens. The calculation of the plasma calcium concentration, in mg %, was performed by comparison with the mean value found for the calcium standard titrations. The oxalic acid titrations were used as an additional control of the standard estimations.

The *error of the method*, calculated as the standard deviation of a series of 20 separate titrations of the same blood sample, was found to be only 0.08 mg % (0.8 per cent of the mean). The error of the standard estimations (on the standard solution) was also found to be 0.08 mg %, as calculated from the great number of double determinations carried out. This error is considerably smaller than that found by ROTHLIN and VON BIDDER (1945). The error of the mean obtained after correcting the titration values of the blood samples with the standard titration values was calculated in accordance with the formula given on p. 107.

Isolated plasma titrations sometimes gave values deviating considerably in both directions from the mean value obtained. In doubtful cases they were tested by the criterion of CHAUVENET (see MELLOR, 1939) before any elimination from the final statistical treatment.

The Phosphorus Method. The method employed was that of FISKE and SUBBAROW, with the modifications of TEORELL (1931). The amount of phosphorus determinable by this method is 10—50 μ g. The following procedure was applied.

From the freshly centrifuged blood sample 0.5 ml of the heparin plasma was diluted with an equal amount of water and treated with 2 ml of 10 per cent trichloroacetic acid, and the mixture well shaken. After 10 minutes 3 ml of water were added, and the mixture was filtered. From the filtrate 4 ml were transferred to a 25 ml volumetric test tube. Water was added, followed by 1.5 ml of 12.5-N sulphuric acid and 2 ml of 5 per cent ammonium molybdate solution and water to the 25 ml mark. Then 0.5 ml of the reducing agent was added and mixed by inverting and shaking. The reducing agent was 0.25 per cent 1:2:4-aminonaphtholsulphonic acid in 14.5 per cent sodium meta-bisulphite and 0.5 per cent sodium sulphite, and was freshly made for each period of analysis. The colour of the test was compared after 15—40 minutes with a control solution without plasma in a photoelectric colorimeter, using red light filter (S 72).

Standardization of the readings was performed at short intervals by a dilution series of a standard phosphate solution. The calibration factor obtained from the series was further checked daily by measuring 4 or more specimens of a standard phosphate solution of 5 mg %, treated in the same manner as the plasma samples.

The *error of the estimation* of both plasma and standard solutions, computed as the standard deviation of 20 separate determinations of the same blood sample and from a large number of double determinations of standard solutions, was found to be 0.13 mg %, or 2.5 per cent of the mean value.

The Phosphatase Methods. During the series 2—4 (March—Oct. 1946) the method of LUNDSTEEN and VERMEHREN was employed (see VERMEHREN,

1938), with some modifications. By this method the phosphatase activity is estimated by the amount of inorganic phosphorus liberated from an alkaline-buffered solution of sodium beta-glycerophosphate, used as substrate for the phosphatase. Unfortunately it was impossible to procure this substrate for the last two series of analyses. Then, the method of KING and ARMSTRONG, in the modification of BUCH and BUCH (1939), was employed. The substrate for the phosphatase is here phenol phosphate, and the liberation of phenol is determined by use of the phenol reagent of FOLIN and CIOCALTEAU. The procedure employed was in accurate accordance with the description of BUCH and BUCH (1939).

The *method of LUNDSTEEN and VERMEHREN* was applied in the following manner. 0.1 or 0.2 ml of the heparin plasma was diluted with 8 ml of 0.9 per cent sodium chloride solution. After shaking, 2 ml of the diluted plasma were transferred to 2 ml of the buffered glycerophosphate solution, made in accordance with the description given by VERMEHREN (1938). The tubes were thoroughly shaken and stoppered and kept in a water-bath at 37° C for 24 hours. Double determinations were usually made of each sample. The hydrolysis was stopped after the 24 hours by adding 4 ml of 10 per cent trichloroacetic acid to each tube. After 10 minutes the tubes were centrifuged or filtered, and 7 ml of the clear filtrate was taken for phosphorus analysis according to the method previously described. The readings were compared with control solutions without plasma, treated in the same manner as the tests. In this way the amount of phosphorus in the test volume was measured, and the corresponding amount of phosphorus in mg per 100 ml of plasma was calculated. From this value the content of inorganic phosphorus of the plasma itself was subtracted (about 4 mg per 100 ml). The rest is then the amount of phosphorus hydrolysed per 100 ml of plasma, and is called the number of "phosphatase units". For children the values were in the range 100—200 "units", and for adults about 50 "units", which is in agreement with the normal values given by VERMEHREN (1938).

In the *experimental control* of the method it was demonstrated, that the rate of the hydrolysis was constant during the 24 hours incubation period. It was also shown, that the phosphatase analysis of heparin plasma did not give significant differences from the values found by using serum.

The *error of the method*, calculated from the large number of double determinations carried out, was 10 "units", or 7 % of the mean. In a mean value of 20 single determinations on different individuals, the error due to the method is then only about ± 2 units.

The *phenolphosphate method* in the modification of BUCH and BUCH includes phenol determinations of the test solution and of a control solution of the same blood sample, where the enzyme has not had time to assert an action. The difference between these values permits the calculation of the number of "phenol units" per 100 ml of plasma or serum. It was found that the standard deviation of a single reading in this method was also ± 7 per cent of the mean for both the test and control solutions. It was also found, that the control readings did not differ significantly between different (healthy) individuals. Then, by using for the subtraction the average of the large number of control readings, in-

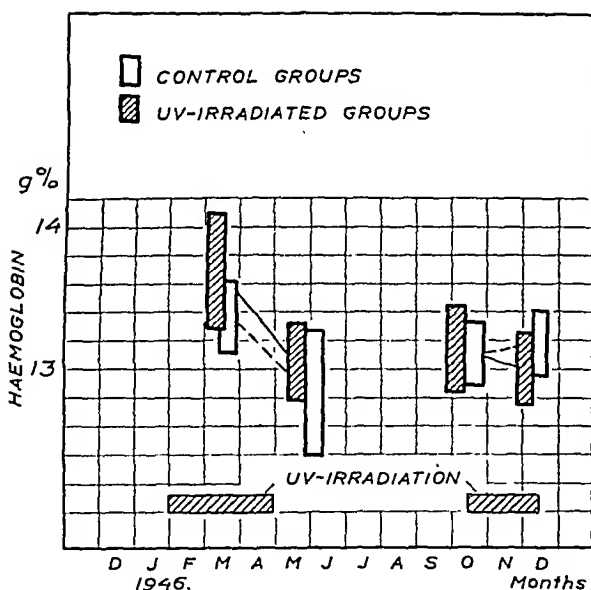


Fig. 30. Haemoglobin levels in irradiated and control groups of Primary school classes.¹

stead of the individual control readings the error of a single determination could be practically maintained at ± 7 per cent.

This method was used only to detect any differences between the groups in each series. The values found are therefore given only as percentages of the (non-irradiated) control groups, and are unrelated for the two time-series, where the method was employed, and also for the values of the previous method.

Results.

The results obtained for the variations of the blood constituents analysed are given in the diagrams of figs 30—38.¹ As discussed above (p. 108), a statistically significant difference between two means obtained for different groups (i. e., uncorrelated samples) is indicated when the corresponding rectangles in the diagrams are mutually displaced so that a free space appears between them. A seasonal variation for one and the same group requires in general a smaller displacement of the rectangles to give the same degree of significance. The exact evaluation of this must be made with consideration to the correlation between the samples.

¹ In the following diagrams the heights of the rectangles denote the range of variation within which the *real* mean value of the group is to be situated with an evaluated probability of 90 per cent (see p. 108). The width gives an estimate of the number of observations in each group (usually about 25, see Table 9, p. 110).

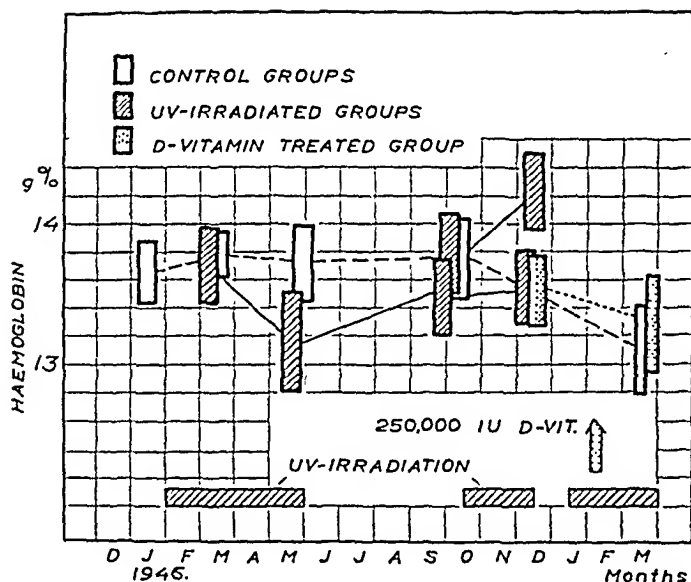


Fig. 31. Haemoglobin levels in experimental and control groups of Secondary school classes.

For the first period, December 1945—May 1946, the control group (i. e., open rectangles in the diagrams) of the Secondary school children has been made up of Group II and the boys of Group III, thus consisting of only boys in conformity with the experimental Group I (see fig. 29, p. 105). The girls of Group III during this period were not included in the statistical treatment. From the following term (Sept. 1946) Groups I—III consisted only of boys aged 10—11 years.

The experimental Group IV of the Primary school children with the corresponding control Group V consisted of both boys and girls, aged about 7 years, during the whole investigation, and the statistical treatment has been made on the combined material.

Effect on Haemoglobin Concentration.

The variations of the haemoglobin concentration of the blood found in the different groups during the investigation are summed up in diagrams, figs 30 and 31, for the Primary school children and the Secondary school children, respectively. In the main there are no differences between the mean values of the irradiated and non-irradiated groups, in spite of the former showing some small deviations in some of the series. These differences cannot, however, be given much significance, due to the lack of uniformity in the response,

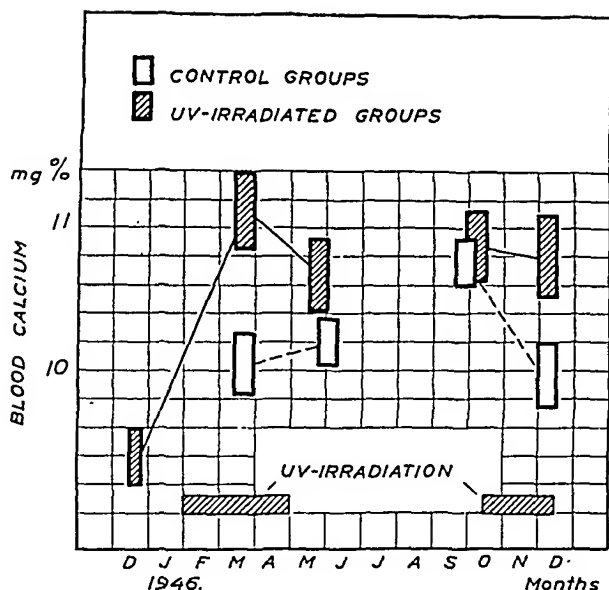


Fig. 32. Plasma levels of total calcium in irradiated and control groups of Primary school classes.

also within the same time-series of estimations.¹ The findings thus indicate that *a continuous low-intensity ultraviolet irradiation of healthy children during the winter does not exert a significant influence on the haemoglobin concentration of the blood.*

That the presence of ultraviolet radiation is not an essential factor for the maintenance of the haemoglobin level in the blood is further supported by the *absence of a uniform seasonal variation* of the haemoglobin concentration in this material. If the ultraviolet radiation were a specific factor for the formation and the maintenance of the haemoglobin content of the blood it would be expected that the values of the control groups would decrease during the course of the winter and increase during the summer. This is not indicated by the present material.

The administration of a massive dose of vitamin D to the non-irradiated children of Group III (fig. 31) did not significantly affect the haemoglobin level (see p. 121).

The failure of an effect of the ultraviolet irradiation on the haemoglobin content of the blood of these healthy school children agrees

¹ The comparatively high mean values found in the Primary school groups in the analysis of March 1946 (fig. 30) may be due to a systematic error in the estimation. The visual photometer readings were in this case performed by another person than in the later series.

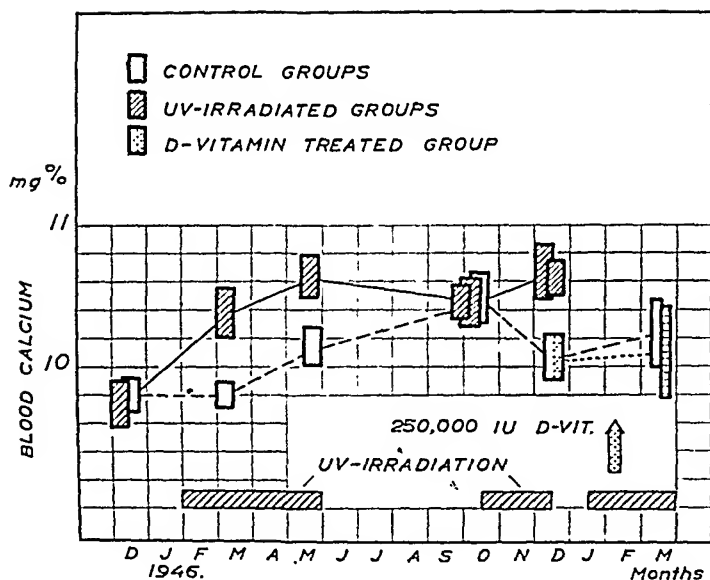


Fig. 33. Plasma levels of total calcium in experimental and control groups of Secondary school classes.

with the now generally accepted view that radiation may be a secondary, additional factor for the restoration of the haemoglobin concentration and the red blood cell count of the blood in cases of secondary anaemia, *the primary factor for the maintenance of normal values, however, always being adequate nutrition* (LAURENS, 1933, 1938; MAYERSON, 1935; Handbook of Physical Medicine, 1945, p. 285). The conception previously prevalent regarding the darkness of the winter as the cause of a seasonal variation of the haemoglobin content of the blood in infants, with a minimum concentration in spring, is now in the main replaced by the view that this possible variation depends upon the increased frequency of infections during the winter (BARENBERG, FRIEDMAN, and GREEN, 1926; FAXÉN, 1937; see also p. 31). Vitamin D deficiency does not itself cause anaemia, and anaemia combined with rickets is not cured by antirachitic treatment (BAUMAN, 1928, cit. MAYERSON, 1935; see also SCHÖNFELD, 1939).

Effects on Calcium and Phosphorus Metabolism.

The plasma level of calcium.

From figs 32—33 it is apparent that the ultraviolet irradiation caused a *marked increase of the calcium level* of the plasma, significantly

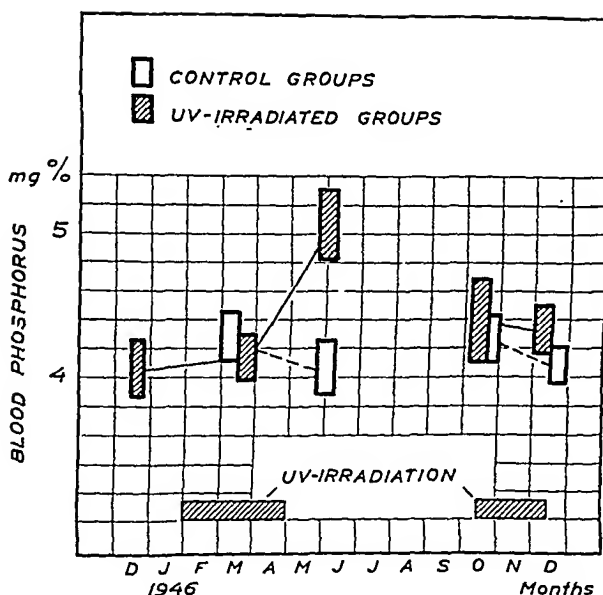


Fig. 34. Plasma levels of inorganic phosphorus in irradiated and control groups of Primary school classes.

occurring in all the irradiated groups during the winter months. The elevation was especially marked in the irradiated Primary school class (Group IV) during the first term, when the ultraviolet intensity in the illumination was highest.

It is also clearly demonstrated that the untreated control children exhibit a *uniform seasonal variation* of the plasma calcium level, with the maximum values in the autumn and the minimum values in the late winter months. The amplitude of the variation seems to be somewhat larger in the groups of the younger children. *By means of the UV-illumination system employed in the classrooms this seasonal variation is cancelled, with the calcium concentrations maintained at the height of the normal autumn level.*

Single values as high as about 12.5 mg % were found in some of the irradiated groups during the winter and in all groups at the autumn analysis. There is no reason to assume that the calcium levels reached by the artificial irradiation fall above the normal upper limit of it. The standard deviation (σ) of the samples varied between 0.3 and 0.6 mg % in both the irradiated and control groups, and with an average of about 11 mg %, as found in the autumn analysis, single values should be expected to reach the range about $(11 + 3 \cdot \sigma)$ mg %, i. e., about 12.5 mg %, due to the random distribution.

In the groups containing children of both sexes it was noticed in 9 out of 11 such samples that the calcium concentrations of the plasma were higher among

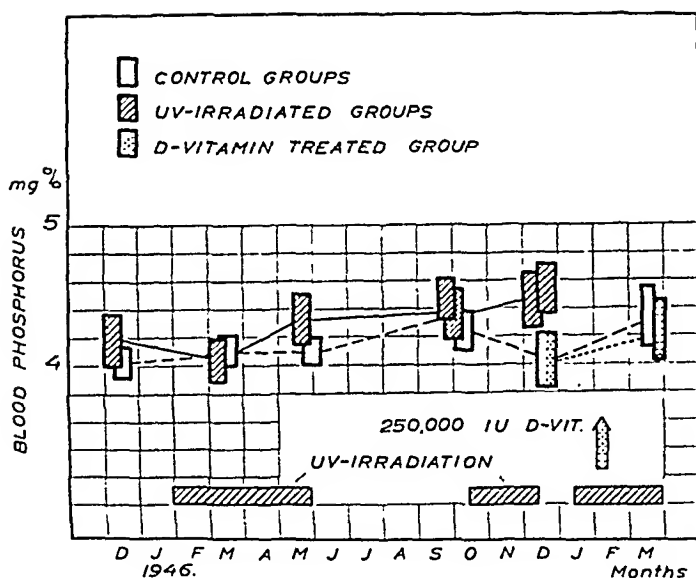


Fig. 35. Plasma levels of inorganic phosphorus in experimental and control groups of Secondary school classes.

the girls. The difference was statistically significant in some of the series, but the mean difference between girls and boys during the whole period of the investigation was only 0.2 mg %.

The plasma level of inorganic phosphorus.

The variations of the plasma concentration of inorganic phosphorus is seen from figs 34 and 35 for the groups of Primary and Secondary school children, respectively. The effect of the irradiation was not at all so pronounced as for the calcium concentration, but *some increasing effect during the winter months seems probable*. The significance of the difference found between the combined irradiated Groups I and II and the control Group III in the analysis of December 1946 (fig. 35) corresponds to a probability of about 0.01. The comparatively very high mean value obtained in May 1946 in the irradiated Group IV (fig. 34) was checked by double determinations on about half of the same children, after an interval of one week. It should be pointed out that this sudden increase of the phosphate level occurred *after* the irradiation had been removed for about one month.

It is also seen from the figures that the results *do not indicate any pronounced seasonal variation* of the plasma inorganic phosphate concentration in this material. As for the haemoglobin variations

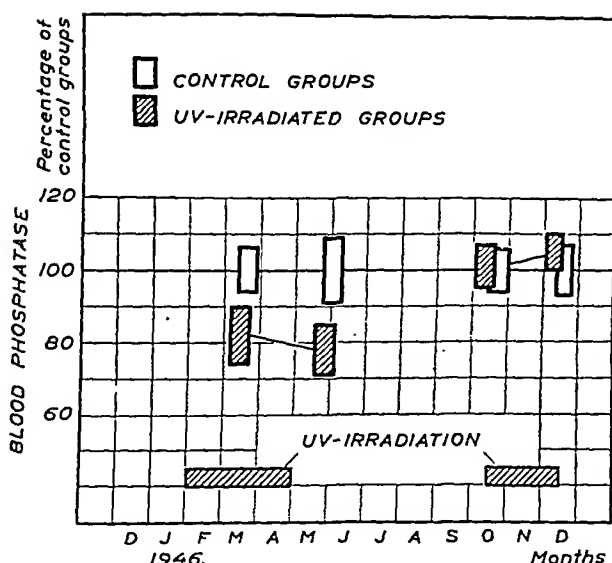


Fig. 36. The relative levels of the plasma activity of alkaline phosphatase in irradiated groups compared with control groups in the Primary school classes.

the failure of a marked effect of the artificial irradiation during the winter in combination with the absence of a seasonal variation may be taken as evidence that ultraviolet energy is not the main factor influencing this blood level in children of school age.

The phosphatase activity of the plasma.

In figs 36 and 37 for the Primary and Secondary school children, respectively, the 90 per cent probability range of the means of the non-irradiated control groups have been plotted around a relative value of 100 in all the series of determinations, and the means of the experimental groups are expressed as the percentages of the corresponding control group. It is seen that in most of the series of analyses carried out during the winter and spring months there was a *significant decrease of the alkaline phosphatase activity in the blood of the irradiated children.*

A measure of the *seasonal variation* of the phosphatase activity was, for methodical reasons, only obtainable during the period March—October 1946, including three series of estimations. The only children followed consistently over this period were those of Groups I and II, and the values found for them by the method of LUNDSTEEN and VERMEHREN are summed up in fig. 38. The marked decrease of the

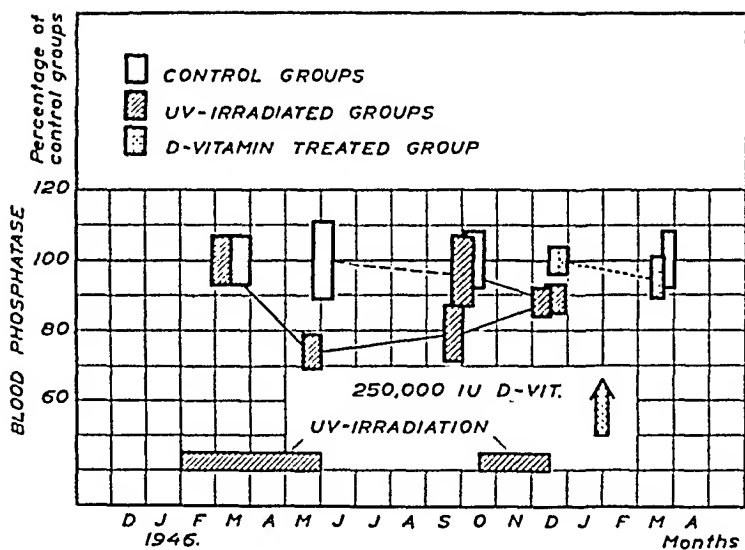


Fig. 37. The relative levels of the plasma activity of alkaline phosphatase in experimental groups compared with control groups in the Secondary school classes.

blood phosphatase activity normally occurring during the summer season is demonstrated, and it seems reasonable to assume that *the low summer value can be maintained by the continuous ultraviolet irradiation during the winter.*

The Vitamin D Experiment.

In order to study if the blood effects observed with the children of the UV-illuminated groups during the winter could be obtained simply by a *supplementary administration per os of vitamin D*, the following preliminary trial was made.

For several reasons, however, it was not possible to organize this trial on a scale sufficient to be expected to yield significant and reliable results. The only possibility left was to give a massive dose of pure vitamin D to a limited number of the control children at mid-winter and study the effects of this. A better treatment of the question would obviously have been to provide for a continuous and sufficient supply of vitamin D during the whole winter, and to use a material sufficiently large to permit general conclusions. The intentions of this trial are only indicative.

At the beginning of the spring term 1947 (Jan.—Feb.) a dose of 250,000 I. U. of vitamin D₂ (calciferol in oil; “Ultranol fortior”, containing 250,000 I. U. per ml) was given to every second boy of the

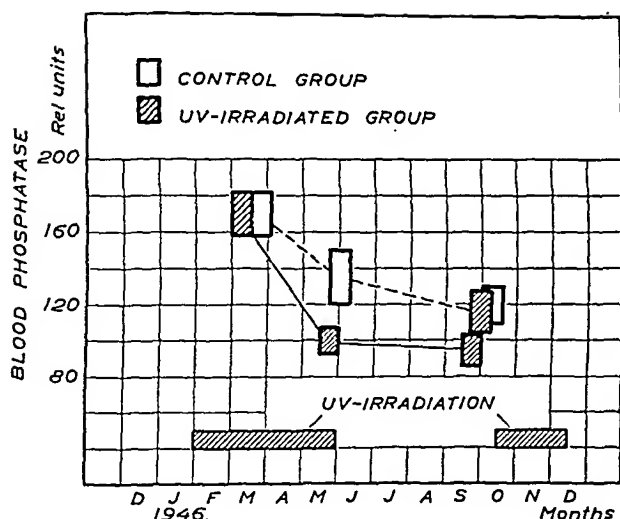


Fig. 38. Seasonal course of the plasma activity of alkaline phosphatase in the Secondary school classes for the summer half-year.

control Group III (aged about 11 years). The vitamin was administered *per os*, mixed in a small amount of milk, and divided into two equal parts given with an interval of two weeks. The other boys of the class were given at the same times some paraffin oil in the milk, unconscious of the difference. After about one month new blood samples were taken and analysed as before with regard to the concentration of calcium, inorganic phosphorus, alkaline phosphatase and haemoglobin. The results obtained are shown by the pointed rectangles in the previous diagrams. It is seen that there was *no significant effect of the vitamin D administered in any of these respects*.

With regard to the small sample studied and to the manner of administration employed it would, however, be wise to postpone any decisive statement on this question until further experiments are carried out, with larger samples and with a continuous administration of the vitamin during the winter. The failure of an effect on the blood constituents should be compared with the effect obtained on physical fitness (p. 144).

Discussion.

It is shown that the calcium and phosphorus metabolism, as reflected primarily by the plasma concentrations of total calcium and alkaline phosphatase, is subject to a *seasonal variation* in Swedish

school children of 7—10 years of age with a presumed adequate diet. The *plasma level of calcium* showed a decrease during the course of the winter with the minimum values reached about March, and an increase during the summer to a maximum value in September or October. The activity of the *alkaline phosphatase* in the blood varied according to a seasonal curve which is the inverse of that for the calcium level, i. e., with the maximum value in spring and the minimum value in autumn. The level of *inorganic phosphorus* in the plasma remained almost unchanged during the course of the year, and the same was the case with the haemoglobin level.

The variations of the calcium and phosphatase levels in the blood were completely *counterbalanced by a continuous low-intensity ultra-violet irradiation* of the children during the winter, applied by means of the UV-illumination system in the classrooms. The values were maintained at the normal autumn levels, which may be regarded as constituting the optimal condition for the calcification of the growing bones. There was also some increasing effect of the irradiation on the plasma concentration of inorganic phosphate, but this was not so marked as for the calcium and phosphatase concentrations. Thus, *the effects of the artificial irradiation during the winter were first seen with those functions which otherwise exhibit a marked seasonal variation.*

It seems reasonable to assume that these effects of the UV-illumination system are due, in the first place, to a *photochemical formation of vitamin D and related substances in the skin of the irradiated children.* The mechanism of the vitamin D action on the human body is, among other things, to increase the net absorption of calcium from the intestine (NICOLAYSEN, 1937; GREENBERG, 1939, 1945), while the effect on the phosphorus absorption is less pronounced (COHN and GREENBERG, 1939). An early symptom of vitamin D deficiency in infants is the increase of the activity of alkaline phosphatase in the blood, and the elevated phosphatase values are brought back to the normal values by administration of vitamin D (see FOLLEY and KAY, 1936.)

The vitamin D experiment carried out in this investigation, with the administration of a massive dose of calciferol (250,000 I. U.) to non-irradiated children in mid-winter did not, however, reproduce the irradiation effects on the calcium, phosphorus and phosphatase levels in the blood. With regard to the small sample treated and the mode of administration of the vitamin too much significance cannot, however, be attached to this isolated result.

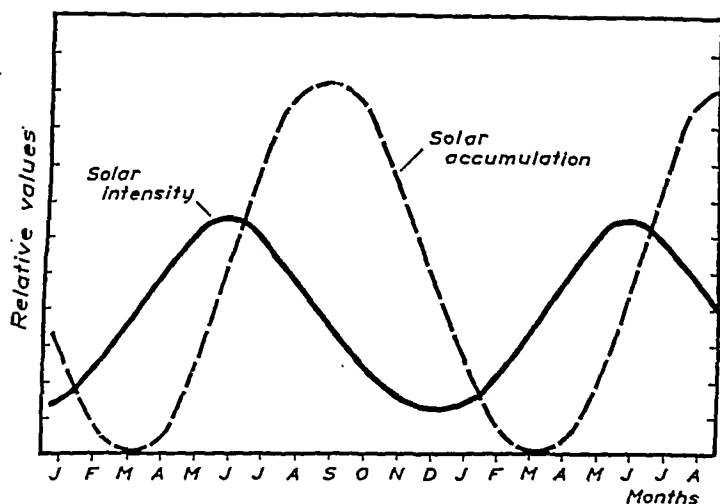


Fig. 39. Theoretically deduced variation of the accumulation in the body of solar-formed substances in relation to the variation of the solar intensity. (After HORSTMANN and PETERSEN, 1946).

The seasonal variation recorded for the calcium and phosphorus metabolism follows the annual course expected for the influence of such a substance as vitamin D, which is mainly supplied to the body by the solar ultraviolet radiation and is balanced in the body metabolism over the year. The accumulation of such a substance in the body should increase during the time of the year when the formation is greater than the consumption, that is, during the summer half year, and the maximum storage in the body should be expected around the autumn equinox (23. September in the northern hemisphere) and the minimum storage around the spring equinox (21. March). The annual course of the incidence of rickets and spasmophilia in infants in Denmark is recently shown by HORSTMANN and PETERSEN (1946) on a large material to follow closely such a theoretically deduced annual curve of the vitamin D accumulation in the body (see fig. 39). It is presumed that the rate of consumption is constant over the year and that the amount of the vitamin consumed in the year equals the amount formed by the solar radiation.

The seasonal variation actually recorded for the calcium and phosphorus metabolism of the children thus gives evidence for the fact that the reduction of the solar radiation during the winter was not sufficiently replaced by the normal winter-diet alone to maintain the optimal condition found in summer and autumn, and the important role played by the ultraviolet radiation is directly proved by the UV-

illumination effects. The autumn levels of calcium, phosphorus and phosphatase in the blood may be regarded as optimal, since they are farthest away from the conditions characterizing rickets (cf. SHOHL, 1939).

HEYMANN (1937) found that after a single massive dose of vitamin D the liver and the blood plasma of rabbits maintained antirachitic properties for 8—12 weeks, and WARKANY, GUEST, and GRABILL (1942) have reported that dried powder of human blood serum is antirachitic for three to six months after the administration of 500,000 I. U. of vitamin D. These findings indicate that the storage of vitamin D in the body from the summer sunshine must be considerably reduced during the course of the winter if no other sources of the vitamin supply are present during this season. The very low intensities of antirachitic ultraviolet radiation prevalent in the natural outdoor daylight during the winter at the latitudes of Scandinavia is mentioned previously, and the limited supply of vitamin D by normal foodstuffs is wellknown (see BICKNELL and PRESCOTT, 1946, p. 652). It is obvious that there really exists a latent risk of vitamin D deficiency in humans during the late winter months.

Previous investigations on seasonal variations of the blood content of calcium, inorganic phosphorus and alkaline phosphatase have only been made on babies, i. e., at the age of the infant when it is susceptible to the roentgenological manifestations of vitamin D deficiency. BAKWIN and BAKWIN (1927) reported such a seasonal variation of the calcium concentration of infant's serum from 8.5 mg % in February—March to about 11 mg % in August—September. HESS and LUNDAGEN (1922) and BRUN (1927) have described a similar annual course for the plasma concentration of inorganic phosphate in infants about twelve months old, and VERMEHREN (1938) found a marked decrease of the phosphatase activity in the blood of small infants during the summer. These variations are ascribed by all the authors to the variation of the vitamin D supply due to the seasonal variation of the intensity and duration of the solar ultraviolet radiation.

The question, however, arises whether all the effects of ultraviolet irradiation on the calcium and phosphorus metabolism are due solely to an increased supply of vitamin D, or if there is also a *hypercalcaemic*, "*calcinosis*" effect involved. This would mean that at least part of the rise of the calcium level in the plasma is due to a dissolution of calcium compounds from the bones instead of, or combined with an increased absorption from the intestine. Such a calcinosis effect of ultraviolet radiation, due to the formation in the skin of a substance related to A. T. 10 of HOLTZ, has recently been assumed by BROCKMÜLLER (1940) and PFEIFFER (1942).

An early blood-chemical symptom of a vitamin D deficiency in infants is usually a fall of the concentration of inorganic phosphate in plasma or serum, besides an increased activity of phosphatase, while a drop of the calcium con-

centration occurs first in later stages (see, e. g., ROMINGER, 1939; CLEMENTS, 1946). The most marked effect both of the artificial and natural ultraviolet irradiation of the children was in this investigation found on the calcium level, and the least effect on the inorganic phosphate level. However, the metabolic effects of vitamin D deficiency in older infants, as involved in this investigation, are not so wellknown; the hypophosphataemia is said to be less pronounced than in the common form of rickets, connected with the normally occurring decrease of the phosphate level in blood with age (SCHÖNFELD, 1939). In adults, ultraviolet irradiation during the winter is found by PONTÉN (1939) to cause an increase of the serum calcium concentration to the normal autumn value of 10.5 mg per 100 ml, but without significant effect on the inorganic phosphate concentration. BROCKMÜLLER (1940) found a rise of serum calcium of about 0.5 mg per 100 ml after ultraviolet irradiation ("Höhensonne") of 17 normal adults, and ascribes this effect, as mentioned above, to a photochemical formation in the skin of a calcinosis factor related to dihydrotachysterol (A. T. 10, see HOLTZ, 1934, 1939). The increase was somewhat higher in winter. PFEIFFER (1942) reported the same findings, based on a material of 33 normal adults, and agrees with the theory of BROCKMÜLLER. The effect on the inorganic phosphate in the serum was not studied in either of these investigations. The serum calcium increasing effect of ultraviolet irradiation was noted already by ROTHMAN in 1923.

Among the intermediate products in the photochemical conversion of provitamin D to vitamin D, at least tachysterol is known to have a calcinosis effect, which, however, is only 1/10 of that of A. T. 10 (see ROSENBERG, 1945). The antirachitic effect of these compounds is very small. The amount of A. T. 10 needed to raise the serum calcium concentration is as high as 2 mg per day, while the amount of calciferol, or vitamin D₃, which prevents or cures rickets is only about 25 µg per day (= 1,000 I. U.; see COHN, COHN, and AUB, 1942). It is evident that some part of the provitamin D present in the skin during ultraviolet irradiation remains at the tachysterol stage, and may be converted into more active forms, but it seems doubtful that the irradiation of a small skin area can leave amounts of tachysterol sufficient to give an increase of the serum calcium in this way. Irradiated ergosterol preparations (viosterol) contain about 50 per cent calciferol (vitamin D₂), the antirachitic principle, and some of the remainder is tachysterol (McLEAN, 1941). In a study of adolescent healthy monkeys (*Macacus rhesus*) for the effect of small doses of a concentrated preparation of viosterol, COWDRY and SCOTT (1936) did not find evidence for the calcium level being significantly affected, but a marked rise of the phosphorus concentration was apparent. The doses of the viosterol preparation used were in all cases far in excess of what might be expected to be therapeutic for the monkeys in question.

Over-irradiation of vitamin D results in the formation of toxisterol (substance 248), which is known to cause hypercalcaemia and metastatic calcifications when given in toxic doses. Direct irradiation of the skin with ultraviolet is, however, said never to cause such toxic

symptoms (HOLTZ and v. BRAND, 1932; DODDS, ROBERTSON, and ROCHE, 1934). ROSENBERG (1945) writes: "The deleterious effect observed on the normal organism upon over-irradiation is independent of vitaminization. This suggests that a special protective mechanism exists in the body, probably in the skin, which takes care of the potential effects of an over-irradiation of the provitamin D."

The material of this investigation does not permit a causal interpretation of the changes recorded for the calcium level, as this presumes an exact study of food and excretion, retention and balance of the calcium and phosphorus compounds. It does not seem, however, justified to regard the effects of the ultraviolet irradiation on the calcium level as toxic, but, on the other hand, the possibility can not be excluded that this irradiation effect involves further mechanisms than just the formation of vitamin D. A possible bone-dissolution effect of ultraviolet irradiation could be appropriately studied by means of the radioactive isotopes of calcium and phosphorus, used by GREENBERG and COHN in the studies of the mode of action of vitamin D (COHN and GREENBERG, 1939; GREENBERG, 1945).

CHAPTER 11.

Effect on Physical Fitness of the Children.

Based upon the reports of LEHMANN and SZAKALL (1932 a-b, 1944) on the improving effect of ultraviolet irradiation of man upon the capacity and efficiency for performing physical exercise, which in different ways have been supported by, among others, MOSCHKOWSKY (1936), PARADE and OTTO (1939) and ALLEN and CURETON (1945), it was decided to study the UV-illumination system regarding this effect also. The inherent difficulties in obtaining valid and reliable information on the fitness for physical exertion of children were clearly realized, but the problem was simplified as the main purpose of the study was to look for possible *variations of the physical fitness of the same individuals* during the time of the investigation.

The important choice of the *method of test* was influenced by the desirability of testing large samples of children in short intervals and without too much interference with the school timetable. Any test

of the maximal physical performance (until exhaustion) was considered unsuitable for application to this child-material, as all such tests are influenced too much by the subjective motivation of the individual to give valid and reliable information, and they also involve some elements of risk. The method finally employed was *a set of cardiovascular tests applied during a continuously graded work on a bicycle ergometer*. In the main the procedure employed is based on the method described by LEHMANN and MICHAELIS (1941). The cardiovascular measurements, which were made during the working period, involved determinations of blood pressure and pulse rate, and the following *criteria of fitness* were used:

1. the total amount of work (in kilogram-meters, kg. m) performed until the product of pulse rate and pulse pressure reached the numerical value 10,000 (LEHMANN and MICHAELIS),
2. the pulse rate at 5 minutes after the beginning of the work, when the amount performed was just 1,000 kg. m, and
3. the pulse pressure after 5 minutes.

In different ways these criteria give an estimation of the stress caused by a moderate muscular work on the cardiovascular system of the subject. A large amount of work performed until the product of pulse rate and pulse pressure equals 10,000, and, consequently, low values of the pulse rate and pulse pressure after a moderate standard amount of work, indirectly indicate a high, latent capacity for heavy work, at least in normal healthy humans. The actual values of the fitness-indices obtained by these tests on a population depend, partly, on the muscle mass of the subject and, partly, on the general working efficiency of the body. Significant variations of the fitness of the same individual during short intervals of tests, without involving any special physical training, must, however, be primarily due to a change in the *efficiency of the muscular and cardiovascular system to withstand the stress caused by a sub-maximal physical exercise*. A detailed account and discussion of the method employed is given below.

It was found that in the homogeneous samples of the school classes involved in this study the values of fitness obtained by these methods were distributed in close agreement with the normal random distribution curve. The material has then been treated in accordance with usual statistical methods of normal random samples. The mean values of the groups are evaluated by the *t*-test and reproduced in diagrams as

Table 10. *Scheme of Tests on Physical Fitness. Only Boys.*

Time	Group I	Group II	Group III
Jan.—Feb. 1946	26	23	—
April 1946	21	20	—
May 1946.....	20	24	—
Sept. 1946	21	23	23
Dec. 1946.....	20	23	23
March 1947	—	17	28 ¹

¹ 14 of whom had received vitamin D.

rectangles showing the 90 per cent probability range of the real mean, as was also employed for the results of the blood analyses (see p. 108). It should be noted that the significance of possible differences between two means of the same group on different occasions (seasons) must be evaluated with respect also to the correlation between the samples; the displacement of the rectangles decidedly under-estimate such a significance and may not be used for an estimate of it.

Only the boys of the Secondary school classes (Groups I—III, see fig. 29, p. 105), aged 10—11 years, have been subjects of the investigation. The time scheme and the number of individuals in the six series of tests carried out is shown in Table 10.

The Test Methods Employed.

The Physiological Basis.

The problems concerning measurement and evaluation of fitness for physical exercise have been particularly treated during the years of the recent war, and some of the prominent works carried out are reviewed by SIMONSSON (1944), H. L. TAYLOR and BROZEK (1944), C. TAYLOR (1945), and KARPOWICH (1947). Only some orientating notes will be given here, mainly referring to the specific questions of the present investigation.

The internal human factors influencing *the maximal amount of physical work an individual can perform* in a given situation, as in repeated, constant laboratory tests on his "fitness", may be schematically divided into the following groups:

1. the actual *mass of muscles* available for the work (the morphological factor),
2. the *power and effectiveness* of the muscle contractions (involving a metabolic, biochemical factor),
3. the degree of optimal *innervation and coordination* of the muscles (the central nervous factor),
4. the power of adaptation of the *respiratory and cardiovascular systems* to the increased demand of oxygen (the oxygen-supply factor), and
5. the *psychological factors*.

All these factors may vary considerably between different individuals, thus giving their real, but difficult to estimate grading with respect to the absolute working capacity. *The only defensible a priori criterion of the fitness of a man for heavy physical exercise is, as C. TAYLOR (1944) states, the amount of such work the man can do until exhaustion.* This limit is, however, always a subjective one, and is decisively influenced by the optimal motivation and interest of the subject. Tests based on this criterion require an intelligent cooperation of the subject if reliable estimations should be obtained. It is obvious that such maximal performance tests are applicable mainly in laboratory investigations on selected material and not suited for a field-investigation like the present one on school children.

The way most commonly used for the estimation of physical fitness is, therefore, the application of *sub-maximal performance tests*. In these the response of some physiological factors to moderate physical work is measured and used as an indication of the latent capacity for heavier work. The criteria commonly used in such tests are cardiovascular in origin (as in, for instance, the SCHNEIDER test), but others have also been employed, as the blood concentration of lactic acid after the work, the respiratory exchange and the pulmonary ventilation, etc. *The reliability of all such submaximal criteria must be controlled with respect to the correlation they offer to the real maximal performance.* C. TAYLOR (1944) found in such a study that the most useful and reliable single criterion in sub-maximal tests on working capacity is formed by the *pulse rate after a standard, moderate amount of work*. The correlation found between the pulse rate during walking on a tread-mill and the time-run to exhaustion was — 0.56, while multiple correlation of pulse-rate, pulmonary ventilation in liters/min., and blood lactate concentration in the sub-maximal phase of the test only gave the slightly better value of 0.63. By using special heart-rate curve functions a correlation of 0.90 could be obtained. In maximal work tests, on the other hand, the increase of pulmonary ventilation and related functions is the most reliable objective index of approaching exhaustion (C. TAYLOR, 1942).

When a variation of a cardiovascular criterion is observed on the same subject during the course of repeated tests, and the influence of psychological and environmental factors as well as any significant growth of the muscles can be excluded, the primary reason must be looked for in a variation of either the *motor efficiency* or the *cardiovascular response*, or both. The motor efficiency may be expressed by the *mechanical efficiency coefficient*, i. e., the quotient of the

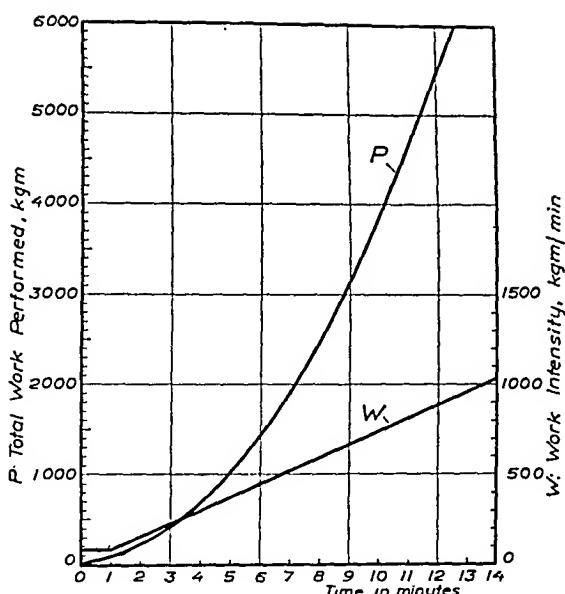


Fig. 40. The grading of the work (work intensity) and the total work performed at different times during the ergometer test.

The reliability of this product for the estimation of the minute volume was first studied by LILJESTRAND and ZANDER (1928). It was found to be an approximately constant relation for the same individual in work tests, the product however rising more and also faster than the minute volume. A somewhat better correlation was found by using the "reduced" pulse pressure, i. e., the percentage of the pulse pressure to the mean blood pressure (see also APÉRIA, 1942). If, therefore, the product used by LEHMANN and MICHAELIS only gives an approximate estimation of the minute volume, the criterion involves the advantage to fitness-tests of also including the pulse-rate function. The latter may of course also be treated separately as a fitness-criterion.

According to the description of LEHMANN and MICHAELIS the test person should work on an ergometer bicycle with the work load continuously increasing at a constant rate. The initial output is adjusted so that the work intensity per square centimeter of the calf cross-section is similar for all the persons. The time until the product of pulse rate and pulse pressure reaches the numerical value 10,000 is the measure taken of the fitness. It is claimed that in this way the method gives a reliable grading of different individuals according to their fitness for physical work.

The main purpose of the work tests carried out during the present investigation was to follow the development of the physical fitness of the same individuals in a rather homogenous sample, without intending to obtain a grading of the different individuals. Because of that no differentiation has been made of the initial load intensity, so that the test has been exactly similar for all the children and in all of the series of tests carried out.

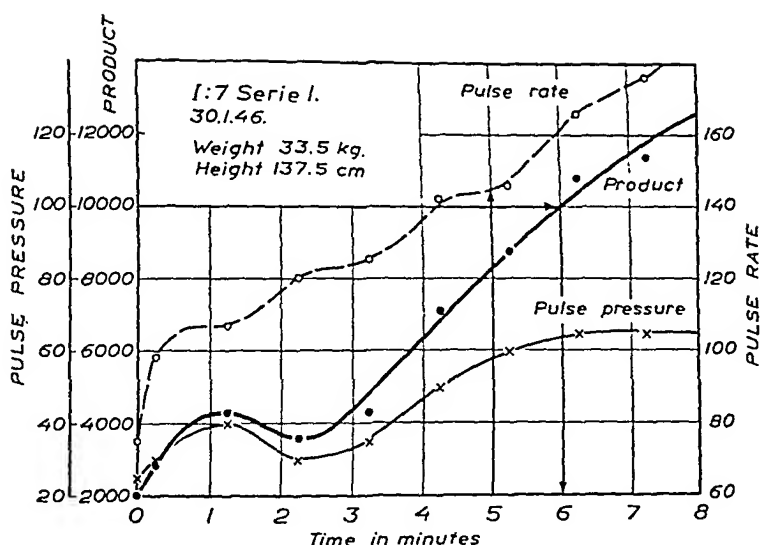


Fig. 42. A typical diagram plotted for a work test on the bicycle ergometer; such diagrams were plotted for each test carried out. — The initial peak occurring after 1–2 minutes is typical, showing the «warm-up» of the subject (cf. WIGGERS, 1945, p. 917).

During the work on the ergometer the blood pressure and the pulse rate were determined every minute. Afterwards, the pulse pressure, i. e., the difference between systolic and diastolic reading, was calculated, and the corresponding product of the pulse pressure and the pulse rate was quickly computed with the aid of the nomogram seen in fig. 41. The values obtained during every test were plotted in diagrams as shown in fig. 42. The time until the product of pulse rate and pulse pressure reached the value 10,000 was obtained by freehand interpolation in the diagram, and the value was converted into kilogram-meters of work performed by use of equation (2) given above. The pulse rate and the pulse pressure at 5 minutes from the start of the test ($P = 1,025$ kg. m) were also obtained in similar ways from the diagrams.

In some of the series carried out attempts were also made to obtain information of the *maximal performance* of the child. He had then to continue the work until he could no longer maintain the pedalling rate. The very subjective character of this «exhaustion» limit was apparent and could be clearly demonstrated by introducing simple encouragements and such like.

The *blood pressure determinations* were made auscultatory with the bell of a membrane stethoscope attached with an elastic bandage over the cubital fossa, below the armlet (width 12 cm). The tubes of the manometer and the pump of the armlet were drawn and fixed to a table besides the ergometer, where the operator had his place. The pressure readings were made with the arm hanging slack, when the armlet was at the heart level. In this way there were usually no difficulties in making exact readings during the first minutes of the work, when the load was still at low values, later becoming more difficult due to the muscle strain and shrugging of the child. However, in almost all cases the readings could be performed with sufficient accuracy until the required

sub-maximal criteria had been obtained. For the product criterion the time value varied for most of the cases between 5 and 8 minutes, while the time to exhaustion was usually 7—10 minutes. The pressure readings were usually corrected to the nearest 5. The diastolic pressure was read at the beginning of the last muffled phase of the disappearing sound ("the fourth phase of KOROTKOW"). The determinations of the pulse rate were made by counting 10 beats heard by auscultation with a supra-diastolic pressure applied.

The tests were always made in the morning between 8 and 11 a. m. and on about 10 boys daily. The temperature and humidity of the environment were always controlled and kept as constant as possible. The great importance of the psychological "atmosphere" during the test was well realized, and all sources of disturbances were eliminated. Great care was devoted to make the boys familiar with the method, and their initial nervousness was usually quickly lost. The tests soon become a kind of sport for the boys, which increased their interest and counteracted any fear of the test. A control of the emotional status was always made by frequent measurements of blood pressure and pulse rate before the start of the test, with the boy placed on the ergometer. All cases of apparent nervousness as well as any case complaining of discomfort or chill are of course excluded from the final treatment of the data.

In the first series of tests carried out (Jan. 1946) the girls of Group III were also included. It was, however, found considerably more difficult to obtain reliable estimations on them than on the boys, mainly due to their nervousness and fear of the test. As the irradiated groups did not contain girls, the following tests were made only on boys.

Undoubtedly it would have also been of value to follow the variations of the resting pulse rate and blood pressure values of the children. To get the true resting value, however, the resting period must be at least 20 minutes (cf. COLLET and LILJESTRAND, 1924 a) and this would have completely spoiled the time scheme of the investigation.

Experimental Control of the Method.

The control of the reliability and accuracy of the tests has consisted of the following studies.

The frequency distribution. When the first series of determinations were made in January 1946, comprising 46 boys and 11 girls, the results were expressed as the time in minutes until the product of pulse rate and pulse pressure had risen to 10,000 (the product criterion). The frequency distribution curves formed by the fitness-values in this unit were, however, rather skew in some of the groups. If the values, on the other hand, were expressed with the total amount of work performed until the criterion was reached as the unit, the distribution curves were usually in closer agreement with the normal distribution curve. The combined material with this unit for the first and the fourth series was tested by the χ -square method with respect to the deviation from the normal distribution curve (see KEMP, p. 112), and no significant deviation was

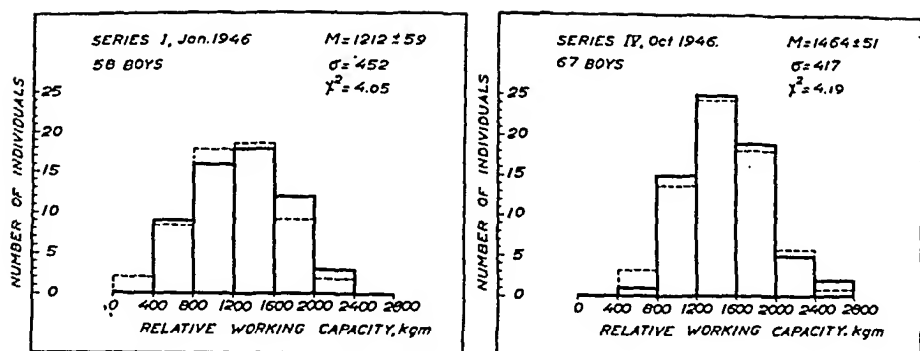


Fig. 43. The frequency distribution of scores of physical fitness obtained by the product criterion (full-line histogram). The dotted lines show the theoretically expected distribution.

estimable (fig. 43). The latter measure was then considered more suitable to use for comparisons between the different samples and series of determinations, as the material could be treated in an ordinary statistical manner.

A statistical analysis carried out at a later stage of the investigation showed, however, that neither did the frequency curve obtained with the time values as unit deviate significantly from the normal distribution curve. This simpler unit may thus be applied as well as the unit used in this investigation.

The pulse rate and the pulse pressure at 5 minutes from the beginning of the test (the pulse rate and the pulse pressure criterion, respectively) were also shown to be distributed in full accordance with the normal curve, thus permitting the usual statistical treatment of the data obtained.

The reliability of the estimations. Three or four tests were made on each of 11 boys at different times of the same day, with the results of the product criterion given in Table 11. It is seen that the morning values were usually the lowest, rising until about noon. The differences are, however, comparatively small in most cases, and seem not to be markedly influenced by the meal taken about noon. The coefficient of variation (V) in the day-series is seen to vary between 5 and 30 per cent, with the mean variability 10 per cent.

Further, double determinations were carried out on 23 boys with an interval of some days, all tests being made in the morning. In most of the cases the maximal performance was also estimated. From these pairs of estimations the retest-reliability coefficient (r_{11}) was calculated for all the criteria used (cf. p. 107), with the results given in Table 12. The reliability coefficient gives an estimate of the random variations of the method and the individual factors affecting the score. To get from this an estimate of the effect of these errors in producing divergencies of obtained scores from their true counterparts, the standard error of a single score obtained ($\sigma_{1\infty}$) is calculated according to the formula (GARRET, 1947)

$$\sigma_{1\infty} = \sigma_1 \sqrt{1 - r_{11}}$$

where σ_1 = the standard deviation of the test scores. This "standard error of

Table 11. *Daytime Variations of Scores of Physical Fitness*
(*Product Criterion, kg.m.*)

Time of Day Subject	8—10 a. m.	10—12 a. m.	13—14 p. m.	14—16 p. m.	<i>M</i>	σ	$\frac{\sigma}{M} \cdot 100$
R.F.	2,600	2,850	2,900	2,650	2,750	147	5.3
G.Ö.	1,200	1,300	1,450	1,350	1,330	104	7.8
G.H.	1,150	1,950	1,350	1,600	1,510	345	22.8
T.J.	1,900	—	2,150	2,000	2,020	126	6.2
B.G.	1,900	1,950	1,950	1,800	1,900	123	6.5
L.O.A.	2,100	—	2,200	2,200	2,170	82	3.8
C.K.	550	750	1,000	—	770	225	29.2
J.D. ¹	1,600	1,600	1,900	—	1,700	173	10.2
B.N. ¹	1,700	1,700	1,500	—	1,630	116	7.1
Ö.S. ¹	1,250	1,250	800	—	1,100	260	23.6
L.T. ¹	1,800	1,900	1,600	—	1,770	153	8.6
Mean:							9.9

¹ Received 4 g of calcium gluconate between the first and second test.
See p. 144.

measurement" may also be expressed as a percentage of the mean of the scores in the group, see Table 12. It is evident that the scores of *maximal performance* obtained on this material are provided with the greatest errors, both relatively and absolutely. The *pulse rate criterion*, in spite of the low reliability coefficient, gives the least effective error of the scores obtained.

In the important question of how high the self-correlation of a test must be to be considered satisfactory, GARRET states that in order to distinguish reliably between the means of two relatively small groups of narrow range of ability the reliability coefficient need be no higher than 0.50 or 0.60. If the test is to be used to differentiate between the individuals in the group, however, its reliability should be 0.90 or more. *Thus, for the purpose of the present investigation, the reliabilities of the fitness tests employed may be considered satisfactory.*

The maximal correlation a given test is capable of yielding between the obtained scores and their theoretically true counterparts may be found by the formula

$$r_{1\infty} = \sqrt{r_{11}}$$

in which r_{11} = the reliability coefficient, and $r_{1\infty}$ = the maximal correlation between obtained and true scores (*index of reliability*). For the product criterion test, for instance, the maximal correlation should be $\sqrt{0.95} = 0.97$; when the means of the day-series reported in Table 11 are taken as estimates of the "true" scores, the correlation between these mean values and the corresponding observations is found to be 0.95 ± 0.02 .

Table 12. *Reliability and Validity of the Fitness Tests.*

Criterion	Reliability Coefficient	Standard Error		Correlation to Max. Performance	
		Absolute Value	Per Cent of Mean	Obtained Value	Corrected Value
Maximal Performance (kg.m)	0.64	470	16	—	—
Product Criterion (kg.m).	0.94	130	10	0.40	0.52
Pulse Rate Criterion (Beats/min.)	0.74	6	4	—0.40	—0.58
Pulse Pressure Criterion (mm. of Hg)	0.80	5	8	—0.25	—0.35

The correlation to maximal performance. The *validity* of every sub-maximal work test for assessment of physical fitness must be controlled with respect to the correlation to the maximal exercise performance. The inherent uncertainties in determining the latter on this special material are mentioned previously, and it may be expected that *the correlation obtained is not the true value particularly due to the error of this variable*. On a selected material of adults C. TAYLOR (1944) found the retest reliability for time-run to exhaustion on a tread-mill to be as high as 0.95, while the corresponding reliability on this material is only 0.64 ± 0.14 .

Estimations of maximal performance were regularly made on all children during the first two series (January and May, 1946), later, after some cases of acute over-exhaustion, only random samples from some of the groups were tested. A total of 146 maximal performance tests have been made, on about 80 boys, and the coefficients of correlation for the different criteria from the whole material are given in Table 12, column four.

As the maximal performance criterion as well as the different sub-maximal tests are both unreliable, the correlation between the tests and their criterion is reduced. In order to estimate the highest correlation the test could theoretically yield between the true scores of these measures, the formula of *correction for attenuation* may be applied, which takes account of the unreliability in both sets of measurements (cf. GARRET, p. 396):

$$r_{\infty} = \frac{r_{12}}{\sqrt{r_{11} \cdot r_{22}}}$$

in which r_{∞} = correlation between true scores in tests 1 and 2, r_{12} = correlation between obtained scores in tests 1 and 2, r_{11} and r_{22} = the reliability coefficient of test 1 and 2, respectively. The corrected values obtained are given in Table 12, last column, and it is seen that *the pulse rate criterion is likely to yield the best estimates of maximum performance*, and the value actually found (—0.58) is in close agreement with the correlation found by C. TAYLOR (1944, cf. p. 130).

It is seen that the pulse rate criterion is as effective as the product criterion in estimating the fitness for heavy exercise even if the correlation actually obtained is used. The pulse pressure criterion has a very small validity in predicting maximal performance.

The relation to body constants. The scores of fitness obtained for every boy by the product criterion have been correlated to body weight, body height, body surface and calf circumference. In this material there was in no case any significant correlation, the highest coefficients obtained were for the body height (0.34 ± 0.09) and the body weight (0.28 ± 0.10). The assumption was made *a priori* that the score per kilogram body weight, i. e., the quotient relative performance (kg. m)/body weight (kg.), could be used as a "*fitness index*" of the subject, indicating the height (m) to which, under the given conditions he could lift his own body weight until the cardiovascular stress rose to the level corresponding to the approximate minute volume factor of 10,000. As, however, the material represented in these series was so homogeneous with respect to the body weight it was considered unnecessary to make any such "corrections". The correlation between the relative working capacity, expressed as kilogram-meters of work performed, and the "fitness-index" in meters was as high as 0.94 ± 0.01 .

Comments. Instead of using the product of pulse rate and pulse pressure for the product criterion, there has also been employed, in some of the series, the product of the LILJESTRAND—ZANDER factor (i. e., the reduced pulse pressure, p. 132) and the pulse rate. The correlation between the scores obtained in this way and the scores obtained by the direct product was however 0.87 ± 0.03 , and the validity was not significantly affected. As the LILJESTRAND—ZANDER factor includes more calculations it was considered more suitable to make use of the simpler direct product.

A practical and reliable indicator of the sensitivity of fitness tests is the influence of a chill (see H. L. TAYLOR and BROZEK, 1944; C. TAYLOR, 1945). A considerable decrease of the score was observed in several cases during this investigation in connection with such unpleasantness of the subject, and one case of convalescence from chicken-pox was also clearly indicated by a remarkably low score.

Results.

The results obtained by the *product criterion* on working capacity are summarized in fig. 44, in the same kind of statistical diagram as was previously used. The ordinate, relative working capacity, gives the amount of work performed until the product of pulse rate and pulse pressure during the continuously graded work on the bicycle ergometer reached the fixed value 10,000. The height of the rectangle shows the range within which the real mean value of the group

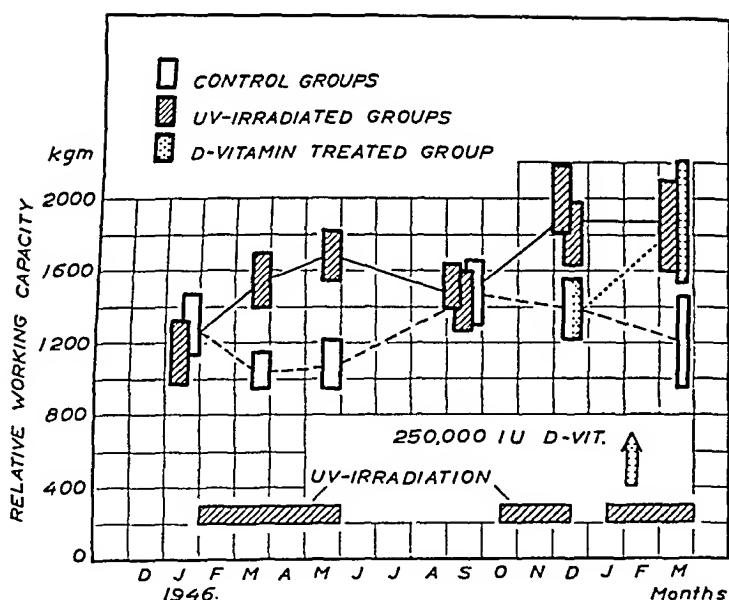


Fig. 44. The seasonal course of the amounts of work performed on the ergometer before the product of pulse rate and pulse pressure reached the value of 10,000 (relative working capacity).

is to be situated with the probability of 90 per cent, as determined with the aid of the *t*-test (p. 108). The number of determinations in every group, approximately indicated by the width of the rectangles, has usually been about 25 (see Table 10, p. 129). The data refer only to 10–11 year-old boys.

It is evident that the ultraviolet irradiation of the children obtained by the UV-illumination system in the classrooms markedly increased the amount of work they could perform before this sub-maximal level of cardiovascular stress was reached. The effect is highly significant; the difference between irradiated and control groups in the late spring months 1946 is 56 ± 7 per cent of the mean of the latter group.

In contrast to the irradiated group, the control group of the first term (Group II, see fig. 29) showed a significant decrease of the score between the first and second series of determinations (January and March, 1946). During the summer months a significant increase occurred. The control group of the following period (Group III, fig. 29) showed, with a high degree of significance ($P = 0.01-0.05$), some decrease between the means of September 1946 and March 1947. The significances of these seasonal variations within the same groups have been evaluated with respect to the correlation between the

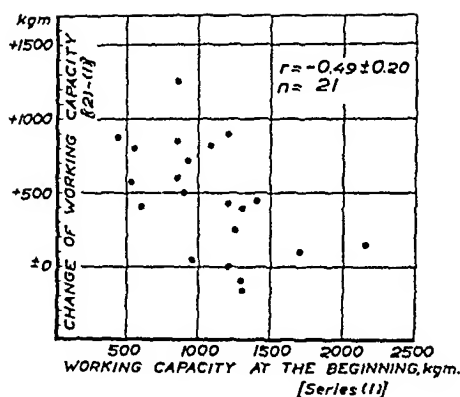


Fig. 45. The relation between the increase of score and initial score (product criterion) in the irradiated class (Group I) during the first months of irradiation.

samples (see p. 109), and they are not clearly estimable from the diagrams.

Thus, it is indicated that the relative working capacity of the control children was subject to a *seasonal variation* with a *minimum during the spring months and a maximum in the autumn*.

This annual course is the one expected if the "solar accumulation" in the body is a dominant factor (p. 124, fig. 39), and the effect of the artificial ultraviolet irradiation during the winter could then be schematically interpreted as a counteraction of a commonly occurring "winter-" or "spring-fatigue" due to the absence of natural ultraviolet radiation. However, an interpretation of the seasonal variation recorded as solely due to solar ultraviolet radiation must be made with great caution; the significant decrease obtained between the first two determinations may also be due to the experience of the test acquired by both the subjects and the operator during these first stages of its application — overlapped in the experimental group by the heavily increasing effect of the irradiation — and the increase during the summer may also be due to the growth of the children or to the physical exercise of the holidays. The decrease of the mean score of the control group during the last three series is only "probable". Thus, the existence of a solar-influenced seasonal variation of physical fitness must not be considered conclusively proved by these findings only, but such an assumption is supported by the obvious effect of the ultraviolet radiation, as well as by the fact that the irradiated group did not show any significant variation during the summer holidays.

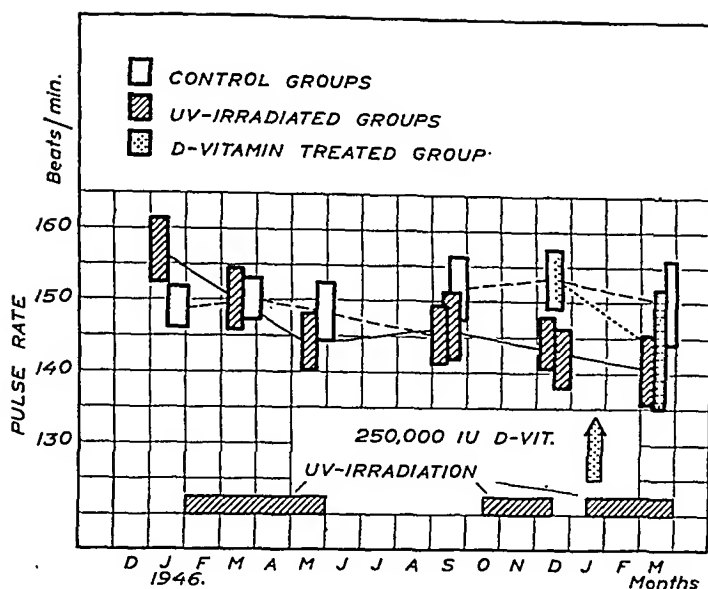


Fig. 46. The seasonal course of the pulse rate after a moderate constant amount of work on the ergometer.

The results of the irradiation during the second period (September—December, 1946) indicate an increase even beyond the normal high autumn level of relative working capacity. The same tendency is also observed when the scores are reduced to score per kilogram body weight ("fitness-index", see p. 139), i. e., the effect may not be attributed to the growth of the children.

In fig. 45 the *increase of score* of each irradiated child, obtained by the product criterion for the period between the first and second administration of the test (Jan. and March, 1946), is plotted against the corresponding *initial score value*. It is indicated that the irradiation primarily affected the children with low initial scores, while children with high initial scores were less if at all affected.

Fig. 46 gives, in a similar way, *the scores of pulse rate* after a standard amount of moderate work on the ergometer (1,000 kg. m performed in 5 minutes). By using this simple but reliable indicator of fitness there is also *clear evidence for a favourable effect* of the irradiation. When the differences obtained for the same group between different series of tests are statistically treated, i. e., when account is taken of the correlation between the samples, a significant decrease of the pulse rate score of the experimental groups is found during both the

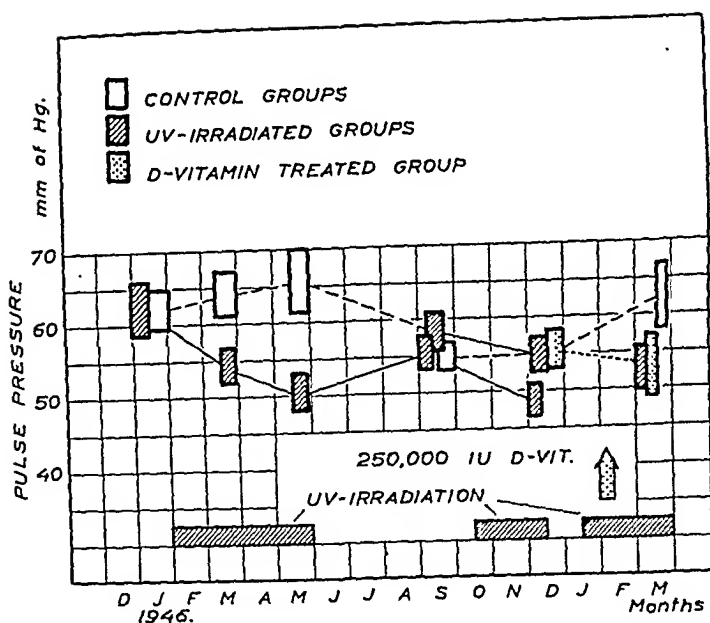


Fig. 47. The seasonal course of the pulse pressure after a moderate constant amount of work on the ergometer.

spring and autumn periods of irradiation. The scores of the control groups remained about constant in all the tests, thus indicating no seasonal variation of the pulse rate score after moderate exercise in normal children.

Finally, fig. 47 gives the variations obtained of the *pulse pressure score*, i. e. the pulse pressure measured after 1,000 kg. m of work had been performed in 5 minutes. The effect of the ultraviolet irradiation applied is apparent, and there is also strong evidence in favour of a seasonal variation of this cardiovascular response on sub-maximal exercise. As a measure of fitness, however, this criterion is less reliable than the others (p. 138). An interpretation of the material has shown that the pulse-pressure diminishing effect of the irradiation or, generally, all the variations observed on the pulse pressure scores, are almost entirely due to the *systolic blood pressure*. The diastolic pressure after the 5 minutes of work was remarkably constant in all the individuals, varying only between the limits 80—85 mm of Hg. It seems probable that the sense of effort and bodily strain during exercise depends, to some extent, on the height of the pulse pressure or the systolic blood pressure, and consequently, the irradiation may have a favourable influence in this respect.

The Vitamin D Experiment.

The effect of the massive dose of vitamin D (calciferol) administered *per os* to every second boy of the control Group III at the beginning of the spring term 1947 is showed by the pointed rectangles in figs 44—47. *It seems highly probable that the same effect obtained by the ultraviolet irradiation was also gained by the vitamin D treatment.* The significance of the difference between the two halves of the class using the product criterion as fitness-measure corresponds to a probability of 0.01—0.001 that the difference would be caused by chance. By using the pulse rate criterion there was also a significant effect of the vitamin D treatment; the mean decrease of the pulse rate for 9 boys tested both before and after the administration of the vitamin was 19 beats/min. with the standard error 3.5, thus giving a critical ratio, t , equals 5.4, which corresponds to a probability < 0.01 for a difference by chance. The reason why this high degree of significance is not so apparent in the diagram, fig. 46, is obviously the high correlation between the individual scores in the two samples. The pulse pressure criterion also showed a highly significant effect of the vitamin D treatment. It should be pointed out that the differences obtained cannot be ascribed to a psychological influence, as the control children were given sham-doses (paraffin oil) in the same manner and at the same times as the experimental children had their vitamin doses.

As in the case of the lack of an effect of the vitamin administration on the blood constituents it must, however, also be emphasized here that the material treated has been too small to permit a general conclusion regarding the influence of vitamin D on the performance of physical work.

A calcium ingestion experiment. In order to study whether an elevated level of total calcium in the plasma may affect the point scores of the test, four out of the eleven boys studied for possible daytime variations (p. 137, Table 11) were given 4 g of calcium gluconate *per os* between the first and second administration of the test. By repeated analyses of the blood an increase of the plasma level of calcium was found by, on the average, 0.5 mg % two hours after the calcium ingestion. There was in no case any effect observed on the score points of the test.

Discussion.

It is shown that a continuous low-intensity ultraviolet irradiation of school children, applied by means of the special UV-illumination system employed, markedly increased the scores of physical fitness of the children, as estimated by a battery of submaximal cardiovascular tests. The effect was particularly pronounced during the winter and spring months when there was a tendency for a decreasing score among the control children. The summer vacation was followed by a significant increase of score for the control children but did not influence the previously irradiated children. Further it has been shown very probable that the same effect obtained by the irradiation is also reached by the administration of a massive dose of vitamin D (calciferol) in the mid-winter.

In interpreting these findings it is suitable to distinguish between the two questions involved, i. e., firstly, the *effect of the artificial irradiation* on fitness and, secondly, the *prevalence of a seasonal variation* of fitness among normal children.

The significance of the irradiation effect is without doubt and the present findings are only to be added to the previous studies carried out (LEHMANN and SZAKALL 1932 a-b, 1944; MOSCHKOWSKY, 1936; PARADE and OTTO, 1939; and ALLEN and CURETON, 1945). An important conclusion from the present study is, however, that *the fitness-improving effect of ultraviolet irradiation may also be obtained by sub-erythral exposures*. LEHMANN and SZAKALL in the later paper (1944) stated, indeed, that an erythema of the skin was necessary for an effect, and the irradiation effect is assumed to be due to erythral substances liberated in the skin. PARADE and OTTO on the other hand assumed the effect to be due to formation of vitamin D, acting upon the adrenal cortex. The vitamin D experiment carried out in the present investigation strongly supports the assumption of a vitamin D action, and the possible role of erythema seems doubtful.

The *hypothesis of a vitamin D action* is of interest to combine with the studies carried out by RÄIHÄ et al. (1937), later extended by PEITSARA (1944), regarding the influence of vitamin D on the metabolic processes and the working capacity of isolated muscles. The importance of phosphorus for the energy-liberating chemical processes involved in the muscle contraction is a well-known fact, as well as the importance of vitamin D for the internal phosphorus

metabolism. There is, however, besides the above mentioned studies, comparatively little work carried out on the influence of vitamin D on muscle work and muscle metabolism, in spite of the apparent muscle hypotoni associated with rickets (for literature see PEITSARA, 1944). In recent reviews of physical performance in relation to vitamins and diet (KEYS, 1943; HENSCHER, 1943), the vitamin D is not even mentioned.

The results obtained by RÄIHÄ et al., and PEITSARA may be briefly summarized as follows:

1. Tetanus-stimulated normal muscles of young rabbits or dogs show a marked increase of inorganic phosphorus and a moderate increase of hexose phosphates during the work. In rachitic muscles the increase of the concentration of hexose phosphates is much greater than in normal muscles, with a corresponding decrease in the liberation of inorganic phosphorus. No difference of the initial concentration of the phosphate-spenders (i. e., phospho-creatinine and adenosintriphosphate) was observed between the normal and rachitic muscles.

2. The administration of a large dose of vitamin D to the rachitic animals immediately (after one day) breaks down this accumulation of hexose phosphates in the muscles. Such a high dose of vitamin D to normally-fed rabbits also counteracts the moderate accumulation otherwise occurring in their muscles during the work. The concentration of inorganic phosphorus is markedly increased in both of these cases.

3. The strength and working capacity of isolated muscles from rachitic dogs are considerably smaller than for healthy dogs (PEITSARA). A large dose of vitamin D restores the working capacity in a striking manner to the normal value, or even higher, the effect appearing after only one day.

These studies indicate that one of the actions of vitamin D in the body is an *activation of the breakdown of ester phosphates* accumulating in the muscles during work, which at least in rachitic animal is followed by an increase of the working capacity of the muscle. That vitamin D in some way favours the conversion of organic phosphorus to inorganic is also reported by COHN and GREENBERG (1939). Some connection may exist between the vitamin D influence and the possible benefit to physical performance derived from high phosphate intakes which has been claimed especially by German authors (see BAUR, 1936; ATZLER, 1939); KEYS (1943) gives a critical review of this particular field.

There are also, of course, other possibilities for the explanation of the ultra-violet irradiation effect on the criteria used in the sub-maximal tests applied. The cause of the action may be sought in each of the five primary factors pre-

viously mentioned as affecting the working capacity of an individual (p. 130); the influence of psychological factors, however, may be regarded as sufficiently eliminated. The possibility of an effect on the cardiovascular system ought to be considered. It has been known for a long time that ultraviolet irradiation causes a decrease of the systolic blood pressure, which effect is usually ascribed to the vaso-dilating effect of liberated erythema substances (JOHNSON, POLLOCK, MAYERSON, and LAURENS, 1936). In spite of a visible erythema of the skin there may be a vaso-dilating action of irradiation on deeper organs, as the muscles, giving better conditions for the performance of work. The increase of the calcium level in the blood caused by the irradiation may affect the nervous irritability and also slow down the pulse rate, thus giving a smaller response on the stress of the work. In fact, MARTIN (1939) has reported that the daily ingestion of calcium gluconate expedites the recovery from exercise, but no convincing data have been presented (see KEYS, 1943). That a single, high dose of calcium gluconate, giving a rise of the plasma calcium level by about 0.5 mg % did not markedly affect the scores of the present tests in the children is previously mentioned.

Regarding the question of a possible *seasonal variation* of the efficiency and capacity for performing physical work the present investigation has definitely shown a marked increase occurring during the summer vacation of the children, but an actual decrease during the winter may not be regarded as conclusively proved, the probability however being comparatively high. *The most rational explanation*, covering all the results obtained on the variations of the working capacity in the children, would obviously be the following. The vitamin D supply of a child's body is continuously decreasing during the winter, when the consumption of the vitamin is greater than its solar formation. This decrease is followed by some decrease of the physical working capacity, and the minimum is noted in the break between winter and spring when the accumulation of the solar-formed vitamin would have its minimum (HORSTMANN and PETERSEN, 1946, cf. p. 124). Artificial ultraviolet irradiation during the winter completely counteracts this seasonal variation, maintaining the capacity at the normal autumn level throughout the year. The same effect may also be yielded by a sufficient administration of vitamin D in the diet, thus indicating that the effect of the irradiation is due to the photochemical formation of vitamin D.

An important consequence of this interpretation is that *a reduction of working capacity, as estimated by cardiovascular criteria in sub-maximal work tests, is a sensitive sign of vitamin D deficiency* and that the supply of this vitamin during the winter is not sufficient to main-

tain an optimal condition for physical work in children. The latter conclusion was also reached by the interpretation of the results obtained for the seasonal variations of calcium and phosphatase levels in the blood, though the active principle in this case may also be other solar-formed substances than the vitamin D.

The comparatively low scores of physical fitness found in spring may be set in connection with the common layman's experience of a "*spring-fatigue*". All kinds of vitamin have been claimed as treatments for this, but vitamin D undoubtedly least of all. The results obtained here indicate indeed that some part of the commonly-experienced body fatigue in spring is *an after-effect of the poverty of solar radiation during the preceeding winter*. The great popularity of a short holiday in the mountains in spring may be considered to originate in some way from the experience of a favourable influence of the intensive solar radiation; even without actual physical exercise, skiing etc., such a holiday is said to be "health-improving". It also seems probable that the general "tonic" effect of artificial ultraviolet irradiation during the winter depends to a large extent on this affect on the physical fitness. Furthermore, many doctors are claiming for a wholesome influence of cod liver oil during the winter in older children also, and one of the reasons may be the experience of an improvement of physical fitness.

To sum up, the investigation has shown that the solar radiation climate of the latitudes of Sweden is not sufficient in the dark season to maintain an optimal condition in the body for performing physical work, the reason probably being the reduction of vitamin D in the body during the winter. The UV-illumination system has proved to be an effective substitute in this respect.

CHAPTER 12

Effect on Air-Borne Bacteria.

The spectroradiometric analysis of the radiation emitted by the medium-pressure mercury lamp employed for the UV-illumination indicated comparatively strong intensities of radiation in the spectral region below 290 m μ , i. e., radiation with high bactericidal effect.

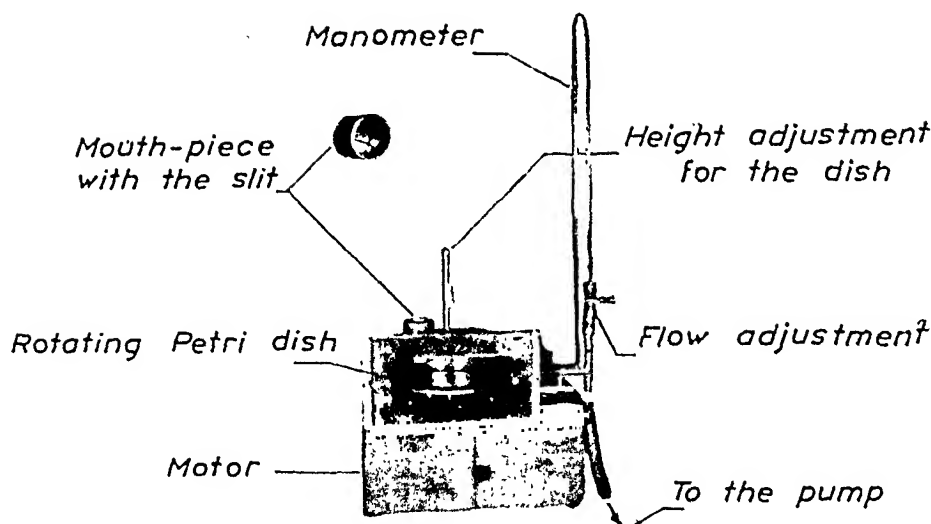


Fig. 48. The air-sampler used for determinations of bacterial concentration.

Most of the energy in this region is concentrated at the wavelength $265\text{ m}\mu$ (see fig. 7, p. 64), which coincides with the region of highest sensitivity of typical bacteria (cf. HOLLÄENDER, 1942).

The investigation of the bactericidal action of the UV-illumination has comprised a laboratory study, made in a small infection-chamber permitting exact control of temperature, humidity and bacterial concentration, and further determinations of the total count of bacteria in illuminated and control classrooms during the lessons. By "total count" is meant the number of colonies which are formed on ordinary blood agar plates by exposure in the air-sampler. Finally, a physical interpretation of the bactericidal effect is carried out, with a comparison between the effect of the partial air irradiation usually employed for the bactericidal, low-pressure mercury lamps and the more uniform irradiation of the room which is obtained with the UV-illumination system.

Method of Air-Sampling.

The concentration of bacteria in the air has been determined by means of an impinging device of the type slit-sampler, constructed at this Institute after an original model designed by BOURDILLON et al. (1941). The apparatus has been previously described by LAURELL, LÖFSTRÖM, MAGNUSSON, and OUCHTERLONY (1947). Fig. 48 shows its features. The chamber contains a slowly rotating table carrying a petri dish with agar, on which the air impinges through a slit in the bottom of the metal cylinder (mouth-piece);

the slit coincides with the radius of the petri dish. The distance between the slit and the surface of agar is adjustable and is indicated by a metal rod which rests on the agar surface (height adjustment). The distance 2 mm was used according to the recommendation of BOURDILLON. The front wall of the chamber consists of an airtight window through which the plates can be changed. The chamber has two outlets, the one attached to a manometer and the other to a suction pump. The rate of flow through the chamber was adjusted to about 35 litres per minute as determined by a flow meter, and it was found that the rate for a given manometer reading was reproducible if the slit was kept perfectly clean. Each plate was exposed for 3 minutes. The nutrient was in all cases ordinary 10 per cent blood agar; no selective culture media were employed. The plates were incubated for 24 hours before counting the number of visible colonies formed during the exposure.

The Infection-Chamber Experiments.¹

The laboratory control of the bactericidal effect of the UV-illumination was carried out by means of a 30 m³ chamber for artificially infected air, with 3 m ceiling height. The chamber was equipped with a complete air-conditioning system which permitted an exact control of temperature and humidity. The air-sampling device was situated outside the chamber and the inlet tube was drawn through the wall so that the sampling could be made from different parts of the chamber. Liquid suspensions of bacteria were sprayed into the chamber by an atomizer and a centrally placed electric fan provided for a homogeneous suspension of the infected air. The injection of bacteria was stopped when the concentration had risen to a suitable level, and the fan was turned off after some minutes. From then the air-sampling was made every 30 seconds. The settling rate of the bacteria was measured in a number of experiments. During the first 10 minutes there was usually a reduction to only about 90 per cent of the initial concentration.

For the tests on the UV-illumination a complete fixture (UV-source + incandescent lamps + reflector) was mounted at ordinary height in the chamber. The illumination was turned on one minute after that the fan had been switched off.

Tests have been made with a number of different bacteria, and

¹ These experiments were made in collaboration with Dr G. LAURELL, Public Health Institute, Department of Public Hygiene, Stockholm. They will be reported more completely in later papers.

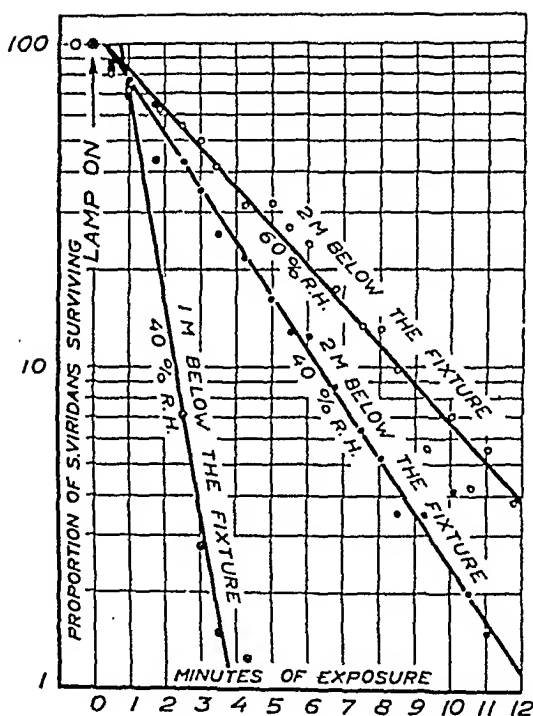


Fig. 49. The reduction of bacterial concentration in the air at different distances below an equipment of the UV-illumination. Test bacterium was *S. salivarius* (*S. viridans*).

a strong bactericidal effect of the illumination was evident in all cases. When using, e. g., *Staphylococcus albus* as test bacterium it was found that the air was practically sterilized in the chamber within the first minute after the lamps had been turned on. The bacterium finally selected for the tests was the more resistant *Streptococcus salivarius* (a variety of α -haemolytic streptococci).

Fig. 49 shows some of the data collected in these experiments. Every dot is the mean of four separate series of determinations made subsequently with reinfected air for every series. Data were in this way collected for relative humidities between 30 and 80 per cent, and with the inlet to the air-sampler placed at different distances and directions from the equipment.

It is seen that the reduction of the bacterial concentration follows an almost straight course when plotted in the semi-logarithmic diagram, i. e., the exponential relationship between survival-ratio and exposure holds approximately for the range of interest for practical purposes. A reduction to 10 per cent of the initial concentration is reached within about 5 minutes in the air 1–2 m below the fixture.

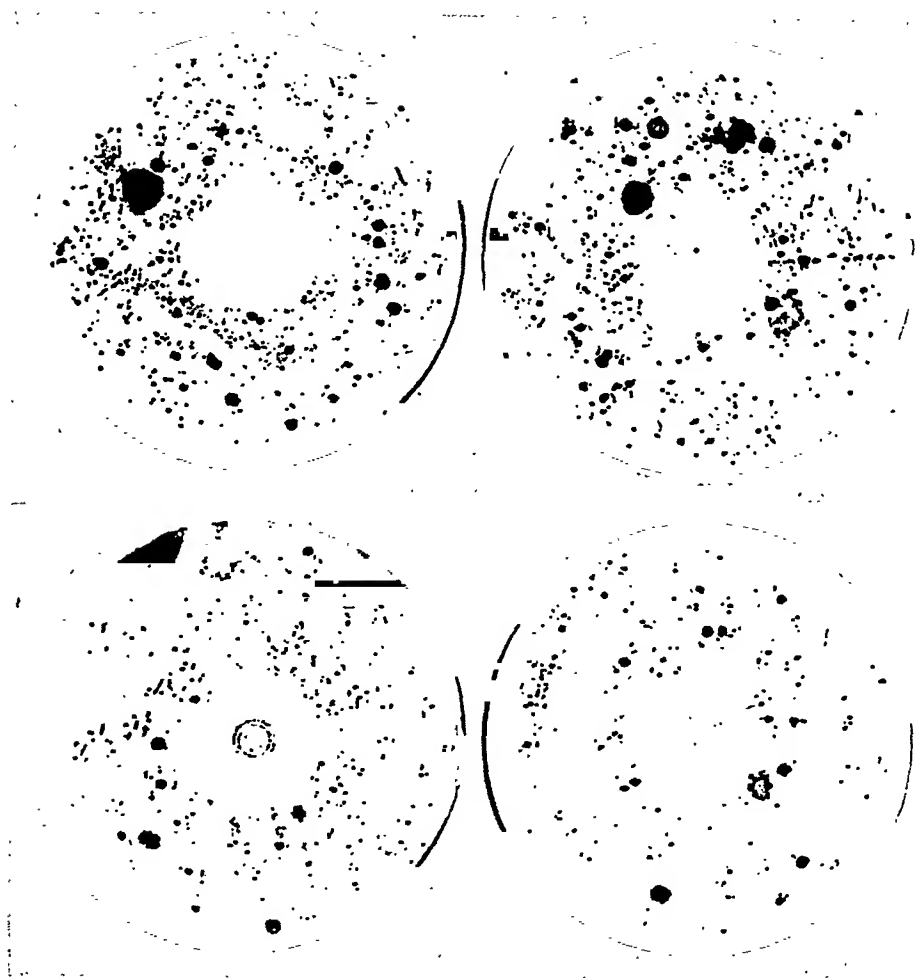


Fig. 50. Blood-agar plates exposed to 100 litres of air in classrooms. The upper two are from a control classroom and the lower two from a classroom equipped with the UV-illumination system.

The killing rate is somewhat smaller in the first minute of irradiation, which is obviously due to the lower ultraviolet output of the mercury lamp before it has reached the normal operating conditions.

The influence of humidity is demonstrated in the figure, and the findings here agree in principle with previous observations (see, e. g., WELLS, 1940).

The Effect in the Classrooms.

Determinations of the concentration of bacteria in the air in classrooms have been made in the spring term 1947 during 20 lessons, 9

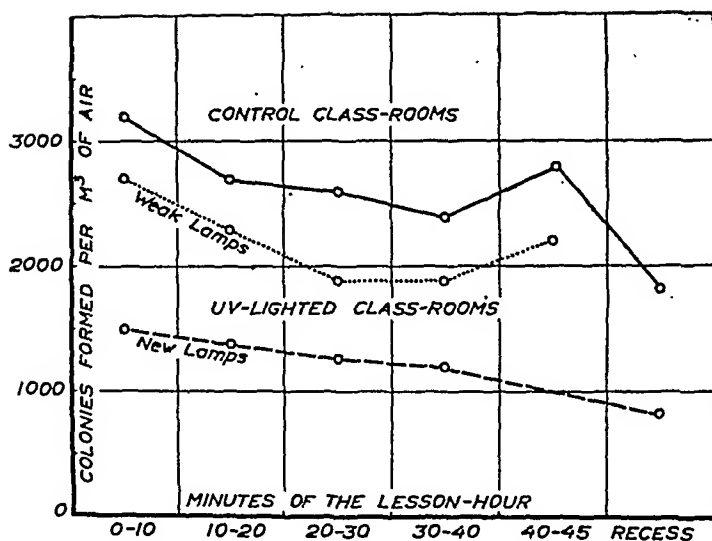


Fig. 51. Mean bacterial counts found for different periods of the lesson-hour in classrooms with different illumination conditions.

of which have been made with the UV-illumination turned on and 11 without UV-illumination. During each lesson about 7 plates were exposed to 100 liters of air for 3 minutes in the air-sampler described above. The samples were taken at 1—1.5 m above the floor among the children. Fig. 50 shows some typical examples of plates obtained in different classes.

The general feature of the bacterial density in the air during the course of the lessons is an immediate and marked increase in the few minutes when the children rush into the room after the break. The concentration may then reach as high as 4,000 bacteria per cubic meter of air, but is usually decreasing slowly during the lesson; the last minutes are often characterized by a new moderate rise. The absolute level is to some extent dependent on the general activity in the classroom; a noisy lesson always gave higher values than a quiet lesson. The ventilation during the recess did not give a complete reduction of the bacterial density; the low temperature outdoors in the winter and spring when the determinations were made did not permit much ventilation. The average counts found for different times of the lesson and for different illumination conditions are given in fig. 51.

In the classrooms equipped with UV-illumination determinations have been made both with and without the illumination turned on.

Table 13. Total Count of Air-Borne Bacteria in Control and UV-illuminated Classrooms during the Lesson-hours.

Classroom	Number of lessons	Number of plates exposed	Mean count per 0.1 m ³	Standard deviation
1. Control.....	11	48	271	63
2. UV, weak lamps	4	24	212	56
3. UV, new lamps.....	5	27	136	53

$$\text{Difference } 1-2 = 59 \pm 14; t = 4.2$$

$$1-3 = 135 \pm 13; t = 10$$

$$2-3 = 76 \pm 15; t = 5.1$$

The concentrations found without the illumination did not differ from the counts found in control classrooms without UV-equipments.

The data given in fig. 51 show an apparent effect of the UV-illumination. The average counts for the lesson-hours found in controll classrooms and experimental classrooms for the whole period of determinations are given in Table 13, with the corresponding statistical data. The differences are clearly significant. *With new ultraviolet sources a reduction to about one half was found for the total bacterial content in the air of rooms with UV-illumination.* With lamps which had burned for more than 300 hours the effect was considerably smaller.

Physical Interpretation of the Bactericidal Effects.

The following account of the physical factors determining the disinfection of air by irradiation is mainly based on the principles developed by W. F. WELLS in a number of papers (WELLS, 1940, 1944, 1945). Other treatises which have been consulted are those by BUTTOLPH (1944) and LUCKIESH and HOLLADAY (1942, see also LUCKIESH, 1946). The interpretation carried out here summarizes, partly in a new form, the basic equations of radiant air-disinfection, but the main purpose is to show the potentialities of the UV-illumination system in this respect, compared with the upper-air irradiation by means of the bactericidal, low-pressure mercury vapour lamps.

The evaluation of bactericidal energy is made by weighting the

spectral intensities of the radiation with the bactericidal action spectrum (cf. Table 1, p. 52). The action spectrum employed is that given by HOLLÄENDER (1942) as typical for most bacteria. The weighted radiation is denoted by UVG.

The Basic Principles.

The following notations are used:

UVG	= ultraviolet radiation weighted by the bactericidal action spectrum,
P_0	= initial concentration of bacteria ($t = 0$),
P	= concentration at the time t ,
t	= time of exposure in minutes,
E	= intensity of UVG in mW/m ² ,
F	= flux of UVG in mW,
V	= volume of irradiated air (m ³),
R	= ray length in the irradiated air volume (m),
U	= unit lethal exposure, mWmin/m ² (see below),
N	= equivalent number of air-changes, and
α	= air-clearance of the irradiation (m ³).

\ln denotes the logarithm to the base e , and \log denotes the logarithm to the base 10.

Surface disinfection. It is repeatedly found that for practical purposes the reduction of bacterial concentration on a surface when exposed to bactericidal irradiation follows an exponential relation according to

$$(1) \quad \frac{P}{P_0} = e^{-kEt}$$

When the dosage Et equals $\frac{1}{k}$ it follows that $\ln P/P_0 = -1$, and $P/P_0 = 0.368$, i. e., this dosage gives a survival ratio of 36.8 per cent of the bacteria. It is called the *unit lethal exposure* or *lethe* (WELLS and WELLS, 1938). In the following it is denoted as U and is expressed in the units mWmin/m². Introducing this entity in equation (1) yields its definite form:

$$(2) \quad \frac{P}{P_0} = e^{-\frac{Et}{U}}$$

Determinations of unit lethal exposure have been made for a number of different bacteria (see HOLLÄENDER, 1942). The values are, for most bacteria, influenced by the humidity of the air or the surroundings. For *E. coli*, which has been most used as test bacterium, WELLS (1945) gives the average value for moderate humidity of 2 μ Wmin/cm² (20 mWmin/m²) of radiation of wavelength 253.7 m μ , and it is found that the sensitivity of ordinary respiratory bacteria in saliva is about the same, or rather somewhat smaller (LUCKIESH, TAYLOR,

and KNOWLES, 1947). Moulds and dustborne bacteria are, on the other hand, 500—1,000 times more resistant. BUTTOLPH (1944) calculates for practical purposes with the unit lethal exposure equal to $10 \mu\text{Wmin/cm}^2$, and LUCKIESH (1946) uses $5.0 \mu\text{Wmin/cm}^2$. The latter value is tentatively applied here as an average for air-borne pathogenic bacteria.

Equation (2) may also be written

$$(3) \quad Et = U \ln \frac{P_0}{P} = 2.303 U \log \frac{P_0}{P}$$

Thus, a reduction from an initial concentration of 100 per cent to the concentration 10 per cent requires about 2.3 times higher dosage as the unit reduction to 36.8 per cent; the reduction to 1 per cent requires about 5 times the value of U (cf. BUTTOLPH, 1944).

Volume disinfection. It is assumed that the bacteria are homogeneously suspended in the air volume (V) and that the radiant flux (F) which enters the volume is uniformly distributed in it and is not significantly depleted by absorption due to either the air or the bacteria. The *ray length* in the volume is R . The volume V may then be considered as built up of an infinite number of differential volumes TdR , thus

$$(4) \quad V = \int_0^R T dR$$

This relation is accurate for a parallel beam of radiation. For a divergent beam, T must of course be replaced by its function of R .

The mean intensity in the volume is determined by the mean surface of the irradiation (T_m) which is found by

$$(5) \quad T_m = \frac{1}{R} \int_0^R T dR = \frac{V}{R}$$

Consequently, the mean intensity (E) in the volume V by the mean ray length R is expressed by the general formula

$$(6) \quad E = \frac{F \cdot R}{V}$$

When applied to a practical case of air irradiation in a room both R and F may be different in different spaces of the room and at different angles to the source. The mean intensity is then obtained as the average of the intensities in the partial volumes ΔV of the room, for which equation (6) may be considered approximately valid, thus

$$(7) \quad E = \frac{\sum (\Delta F \cdot R)}{\sum \Delta V} = \frac{\sum (\Delta F \cdot R)}{V}$$

The units of E for the volume are obviously $\text{mW} \cdot \text{m}/\text{m}^3$ which form the general expression for the bactericidal intensity of the radiation in a volume (cf. LUCKIESH and HOLLADAY, 1942).

The reduction of the bacterial concentration in the volume is obtained by substituting this expression of E in eq. (2), thus

$$(8) \quad \frac{P}{P_0} = e^{-\frac{F \cdot R}{U}}$$

The exponent may be transformed according to

$$(9) \quad \frac{P}{P_0} = e^{-\frac{F \cdot R}{U}}$$

The term $\frac{F \cdot R}{U}$ has the units

$$\frac{\text{mW} \cdot \text{m}}{\text{mW min}/\text{m}^2} = \frac{\text{m}^3}{\text{min}}$$

and eq. (9) consequently has the character of the general equation for a dilution rate due to ventilation. The term $\frac{F R}{U}$ corresponds to that ventilation rate which would give the same dilution of the bacterial concentration as is produced by the irradiation of the air. This figurative but eloquent expression for the bactericidal effect of air-irradiation in a room was introduced by WELLS and WELLS (1938) and is termed the equivalent *sanitary ventilation*.

The expression from the exponent in eqs (8) and (9) may be used separately according to

$$(10) \quad \frac{F R}{U V} = N$$

which gives the *equivalent number of air-changes* in the room (N), which produces the same reduction of the bacterial density.

Equation (9) may be written in the form

$$(11) \quad \alpha = \frac{V}{t} = \frac{F R}{2.3 U \cdot \log \frac{P_0}{P}}$$

which permits the computation of the volume of air (α) which can be disinfected to the ratio $\frac{P_0}{P}$ per minute of the exposure; it may be termed the *air-clearance* of the installation. This equation is particularly practicable for the design of irradiation systems in certain cases (see below).

By introducing in the equations above a unit of *bactericidal power* (P_{UVG}) for the sources, based on the principles developed in Chap. 4 (see p. 49) i. e.,

$$(12) \quad P_{UVG} = \frac{F}{U}$$

the formula are still more simplified and practicable. The power-value gives the area ($\text{m}^2/\text{min.}$) which is disinfected to the unit degree (36.8 per cent survival) if all the radiant flux (F) is concentrated on it. The formula given above for equivalent sanitary ventilation and air-clearance are easily transformed in accordance with this unit. The radiant sensitivity of different bacteria may conveniently be expressed by the relation to the average pathogenic bacteria ($U = 50 \text{ mWmin/m}^2$).

From the above it is seen that the physical factors which primarily influence the bactericidal effect of air-irradiation are the flux of bactericidal radiation (F), the ray length in the irradiated volume (R), and the sensitivity of the bacteria (U). The air-clearance produced by the bactericidal irradiation varies directly with the flux and the ray length but inversely as the unit lethal exposure of the bacteria.

Applications in Practice.

The primary assumptions of a homogeneous suspension of the bacteria and a uniform distribution of the radiation in the air, made for the ideal case treated above, are not strictly valid for most cases in practice. The formula derived must, however, form the basis for the treatment here also. There are two general cases which must be treated separately. The one is the *partial irradiation of the air* in a room, which is the method employed for the low-pressure mercury vapour lamps as disinfecting source. The other case is the *uniform irradiation of the air*, which is employed with the UV-illumination as the bactericidal agent.

Partial irradiation of the air. The irradiation of the air above eye-level is the common form for the administration of bactericidal lamps in the control of air-borne infections. Only small intensities of the radiation are allowed below the eye-level, due to the high risk of eye-injury (see p. 54). Ceiling and upper walls must be painted with ultraviolet-absorbing paints (oil paints). The disinfection of the air in the irradiated zone of the room may be treated according to the formula given above. The resulting dilution of the bacterial content in the occupied region is, however, *exclusively determined by the rate of interchange of air between the irradiated and non-irradiated zones*. If there is no circulation of the air the concentration of bacteria in the occupied zone will increase independently of the disinfection taking place in the upper zone.

Determinations of effective air-circulation in different rooms have been undertaken by, among others, WELLS (1945) and LUCKIESH and HOLLADAY (1942, see also LUCKIESH, 1946). It is related to the rate of movement of the air in the room, which is due to its actual replacement through air ducts, windows and openings and to currents set up by differences in temperature, circulating fans, movements of its occupants, etc. Hot radiators of free situation may increase the circulation several times (cf. LUCKIESH, TAYLOR, and KNOWLES, 1947), and it seems probable that cold windows may have a marked effect too.

There are obvious difficulties in measuring air circulation in occupied spaces; the principle employed is usually to measure the reduction of bacterial density actually obtained in the lower zone and from this calculate a factor for the corresponding effective circulation (see WELLS, 1945). In this way WELLS found the effective circulation between the occupied and the irradiated zone, i. e., below and above the seven-foot level (2.1 m) in standard American classrooms to exceed 4 air-changes a minute.

Such a high value of the natural circulation in a room may, however, not be found in all cases. Measurements of the air movements in Swedish classrooms, carried out during this study with the aid of the kata-thermometer according to the technique described by BEDFORD (1946), indicated a mean movement of only 2—3 m/min. at the 2 m level of the room. If the entire volume of the air is considered to be moving in an unbroken stream, rising through one half of the horizontal cross section of the room and descending through the other half, with the velocity 2 m/min., this would produce, in a room with 50 m² floor area, an air-circulation of 50 m³/min. With the volume of the occupied zone equal to 100 m³, this circulation corresponds to only about 0.5 air-changes a minute, or 30 air-changes per hour. It seems further unlikely that all of the air movements should be effective for the interchange between the upper and lower zone. It ought to be mentioned, that the radiators of the rooms during these measurements were hot, but they were shielded in cupboards in the walls with inlets for the air near the floor and outlets for the warm air near the ceiling.

The air-circulation may of course be *raised by means of fans*. The upper limit is placed by the threshold of draught (about 20 m/min. air velocity); with such an effective circulation the possible number of air-changes per minute in the case cited above would be about 5; even higher values may be obtained if the air is forced at higher velocities along walls etc.

Another factor limiting the resulting reduction of the bacterial density in the occupied zone is *the volume of the irradiated zone*. The efficiency of upper-air irradiation increases markedly with increasing ceiling height of the room (see LUCKIESH and HOLLADAY, 1942). It is found that an irradiated stratum of only about 1 foot (0.3 m) below the ceiling is insufficient to influence markedly the bacterial density in the lower part of the room (LUCKIESH and TAYLOR, 1947). The lamps must be placed about 0.5 m above the eye-level in the room in order to protect the occupants. Thus, in rooms of a ceiling height about or below 2.5 m no great effect of an upper-air irradiation may be expected.

A practicable formula for the *design of a bactericidal upper-air irradiation* of a room is offered by eq. (11) given above. The circulation, natural or forced, available in the room is the volume which should be cleared during its passage through the irradiated zone. The known or estimated value hereof is substituted for α in the equation. The value of R is dependent on the size of the room, and the radiant distribution of the reflector and may be estimated from this; the fixtures should obviously be placed so that the ray length is maximal. Then the equation may be solved for F by substituting the values of unit

lethal exposure (U) and desired degree of disinfection ($\log P_0/P$) in the equation. By assuming U equal to 50 mWmin/m² and the degree of disinfection desired as 99 per cent ($\log P_0/P = 2$), the denominator is 250, and F is obtained in milliwatts. For a 200 m³ classroom with an effective mean ray length of 4 m and 2 air-changes per min., the bactericidal flux (F) required for 99 per cent disinfection is then found to be 25,000 mW (25 W). That flux is obtained with four 30-watt germicidal lamps.

The same calculation made with the unit of bactericidal power according to eq. (12) is easily shown to be carried out by the general formula

$$(13) \quad P_{UVG} = \frac{5 \cdot \alpha}{R} K$$

where K stands for the sensitivity relation of the actual bacteria to the «average pathogenic bacteria». In the present case ($K = 1$) P_{UVG} is found to be 500 m²/min. A 30-watt germicidal lamp (total flux of UVG about 7,000 mW) correspond to a power of about 150 m²/min. (cf. Table 2 and 3, pp. 65—66).

Uniform irradiation of the air. The bactericidal radiation of the UV-illumination is distributed throughout the room, as it is combined with the visible light. However, the distribution is not perfectly uniform but determined by the intensity distribution of the reflectors (fig. 11, p. 71) and to some extent by the reflection from different surfaces. The physical interpretation carried out on the bactericidal efficiency of the UV-installations in the classrooms has been made in the following general way.

The average bactericidal intensity was calculated according to formula (7) given above, for both the region of the room above the reflectors and below the reflectors. The flux of UVG (ΔF) was calculated for every 30 degree zone around the reflector according to the method described on p. 84, i. e., from the intensity distribution curve of the reflectors, and with the total flux of UVG given in Table 2 (p. 65). The corresponding ray lengths (R) were obtained for every zone from the actual dimensions of the classrooms; the average for the measurements in four directions was used. In this way the value of $\Sigma(\Delta F \cdot R)$ was estimated to be 470 mW · m per fixture in the lower part of the rooms and 740 mW · m per fixture in the region above the fixtures. The volume of the lower part is 150 m³ and of the upper part 50 m³. The mean bactericidal intensity in the occupied zone below the four fixtures of the room is then 12.5 mWm/m³, and in the upper zone 60 mWm/m³; these intensities may also be expressed as 1.25 μ W/cm² and 6 μ W/cm², respectively, on the mean surface of the irradiated volumes (cf. p. 156).

However, these values give only the intensities due to the *direct radiation* from the UV-equipments. The ceiling and walls in the rooms were painted with ultraviolet-reflecting paints and add further bactericidal radiation to the air. The *reflected radiation* to be considered is that from the upper part of the room, as the lower part with the floor and furnitures may be expected to absorb most of the radiation. The reflected flux of UVG reaching the working plane of the room was calculated by means of the distribution coefficients, as described on p. 75. The coefficient of the indirect component was estimated to

be 0.2 for the UVG-radiation (cf. Table 4, p. 71), which gives a total reflected flux passing from the ceiling down to the working plane of 250 mW of UVG. The mean ray length of this reflected radiation offers considerable difficulties for the calculations. The height from the working plane of the room to the ceiling is about 3 m, but most of the radiation is diffusively passing through the air, and depleted by further reflections on the walls. The trigonometric calculation of the average distance from a point in the working plane to the ceiling, extended to include the rays reflected from the upper third of the walls, gives the value 4.4 m, which was employed for the following estimates. This ray length gives a total of 1,100 mW · m of reflected bactericidal radiation in the room. About two thirds of this radiation is effective in the lower part of the room. These considerations, which probably yield somewhat too small figures, lead to the estimates that the mean bactericidal intensity below the fixtures in the room is about 20 mWm/m³ and above the fixtures 80 mWm/m³. The disinfecting effect of these intensities may be transformed into *equivalent number of air-changes* by means of formula (10); assuming the unit lethal exposure to be 50 mWmin/m² the following values are obtained:

below the fixtures: 24 *air-changes per hour*,
 above the fixtures: 95 *air-changes per hour*.

These figures give the sanitary ventilation produced by the radiation from the fixtures during its passage through the respective volumes. From the hygienic point of view it is the ventilation in the occupied region which is of particular interest. To the figure given above should be added the effect caused by the circulation of the air through the more strongly irradiated upper part of the room, and further the natural ventilation existing in the room. *An estimate gives a probable total value of about 30 air-changes per hour effective for the dilution of bacteria in the occupied region of the room;* at least 25 are due to the UV-illumination system and of these about 8 to the reflected radiation.

These estimates may be appropriately compared with the data obtained in the *infection-chamber experiments*. It was found (see fig. 49) that the concentration of bacteria (*S. salivarius*) in the air 1—2 m below the fixture was reduced to 36.8 per cent of the initial value in about 3 minutes. No reflected radiation was present in this case, but the intensity of direct radiation was comparable to the conditions in the classrooms. The air-change N which would produce the same bacterial reduction, is simply obtained from the formula

$$\ln \frac{P}{P_0} = -N \cdot t$$

The unit reduction ($\ln P/P_0 = -1$) in 3 minutes gives $N = 0.3$, i. e., 0.3 equivalent air-changes per minute, or about 20 equivalent air-changes per hour, which agrees well with the figure derived above.

The reduction of *total bacterial count* obtained in the classrooms cannot be used for more than a rough estimate of the killing effect on respiratory and pathogenic bacteria. The unit lethal exposures for most of the saprophytic, dust-borne bacteria always found in crowded rooms are, as mentioned above,

500—1,000 times higher than those of most pathogenic organisms. *There may be considerable reduction of the respiratory fraction of the total count without influencing to a higher extent the value of the total count.* Thus, WILLIAMS (1947) found, by upper-air irradiation of classrooms in England, a reduction of the total count by about 25 per cent, while the reduction of α -haemolytic streptococci recovered from the air on selective substrate was about 70 per cent. It seems reasonable to assume that the reduction to about 50 per cent of the total count obtained by means of the UV-illumination system was accompanied by a considerably greater reduction of the respiratory expelled bacteria.

A distinct advantage of the UV-illumination is furthermore offered by the comparatively high intensity of bactericidal radiation incident on the lower surfaces of the room (see Table 5, p. 77). A reduction of the bacterial content in the dust collecting at the furnitures and the floor may be expected to result from this.

Discussion.

The experimental data and the theoretical interpretation of the bactericidal effect of the UV-illumination system employed for the classrooms give evidence that *a sanitary ventilation equivalent to about 30 air-changes per hour may be produced in the occupied region of the rooms*, mainly due to the direct effect of the radiation on the air in this region. This bactericidal effect is obtained without hitherto observed risks of injury to the occupants. It is independent of the circulation of the air in the room, although an improved circulation may yield still higher bacterial reduction.

Studies of the dynamic spread of measles in classrooms have indicated that the minimum sanitary ventilation which can control this spread is about 4 m³/min. per pupil (see WELLS, 1945). In an ordinary classroom of 200 m³ with 30 pupils this means a minimum sanitary ventilation of 36 air-changes per hour. The UV-illumination system in the present arrangement offers a considerable part of this threshold sanitary ventilation, and increases the sanitary ventilation about 10 times as compared with the natural ventilation in ordinary Swedish classrooms. It seems possible that a new arrangement will yield still better effect than hitherto obtained (cf. p. 176).

An important field of application for the UV-illumination system as a bactericidal source is *rooms with low ceilings*. It is shown above that an irradiation of only the stratum above eye-level is insufficient to produce a marked improvement of sanitary ventilation in rooms with ceiling height below about 2.5 m. The usual ceiling height in

modern apartments in Sweden is about 2.7 m. It seems likely that the UV-illumination system may be more effective as a bactericidal agent in such rooms than an upper-air irradiation with the bactericidal low-pressure mercury lamp. Suitable applications may be found in many schools, day nurseries etc.

An inherent factor which to some extent limits the general use of UV-illumination is obviously the high wattage. A sufficient air disinfection in classrooms may be obtained by about 120 watts of low-pressure bactericidal lamps (see p. 160), while the UV-illumination consumes a total of about 2 kilowatts. However, when the day lighting in the room is sufficient for the illumination, only the medium-pressure mercury lamps may be burning, consuming in total about 500 watts in a classroom.

CHAPTER 13.

Effect on Absenteeism.

The intentions of the account given here on the influence of UV-illumination on absenteeism due to illness are only indicative. There are only three classrooms which have been equipped with UV-illumination, and the time of observation is reduced to two terms for each of the classes in these rooms. It would be premature to draw general conclusions from this material for such a variable factor as absenteeism from school, if the effect were not exclusively marked; this is, however, not the case.

In order to give a general background of the records obtained during the period of investigation for both experimental and control classes the absenteeism in all classes of 1st grade and 4th grade in Vaksala Secondary School of Uppsala has been recorded for the years 1943—1947. For each school-year and for each grade 4—5 classes (120—150 pupils) are involved in the statistics. The record is made for each week of the terms, and the data are obtained from the registers kept by the respective teachers. The registers are uniform for all classes, and absenteeism due to illness, as the reason pretended, is recorded separately from absenteeism due to other reasons.

The records are made with respect to two measures of illness-

status in the classes: *incidence of illness* and *lost time due to illness*. The second measure includes also the duration of each case of absenteeism. These two measures coincide in the main but there may be some differences also, which will be shown later.

There was from the beginning no differentiation made between boys and girls in the statistics collected here. It was, however, later made clear that the girls showed in general a higher rate of absenteeism than the boys, which must be considered in the correct statistics. All 1st classes treated, including the experimental groups, comprised about equal proportions of boys and girls and the records are sufficiently comparable for that reason. The experimental groups of 4th classes were made up of boys only during most of the investigation. The control material of the statistics given here involved both boys and girls and a comparison between the means must be undertaken with regard to this disproportion. However, the control material involved at least one class of boys only during the experimental periods, and the range of variation given for the means of different classes may be a justifiable basis for the comparison.

Cases of only one day's duration were also included in the statistics as most of such cases are really due to illness and especially respiratory illness, according to the experience of the teachers.

The responsibility of various diseases for the absenteeism cannot be obtained from the registers. In order to get an estimate of the role played by respiratory infections ("colds") for the total illness-rate, specifications of the diseases were requested from the parents of the children in three 4th classes of the Secondary school for one term (the spring term 1946). Altogether more than 200 such specifications were collected, and it was found that 84 per cent of the total number of absenteeisms during that period were ascribed to respiratory infections by the parents. Other diseases were found to be fairly uniformly distributed over the term, and, therefore, *the seasonal variations recorded may be considered as primarily due to epidemics of respiratory infections*. The role of measles, scarlatina, and other children's diseases formed a very small fraction of the total absenteeism in these 4th classes. For other years and other classes there may have been other proportions which, however, could not be taken into account. Single cases of exceptional diseases (accidents etc.) were often specially denoted in the registers and could be excluded.

The data are reproduced in the diagrams of figs 52—55. The inci-

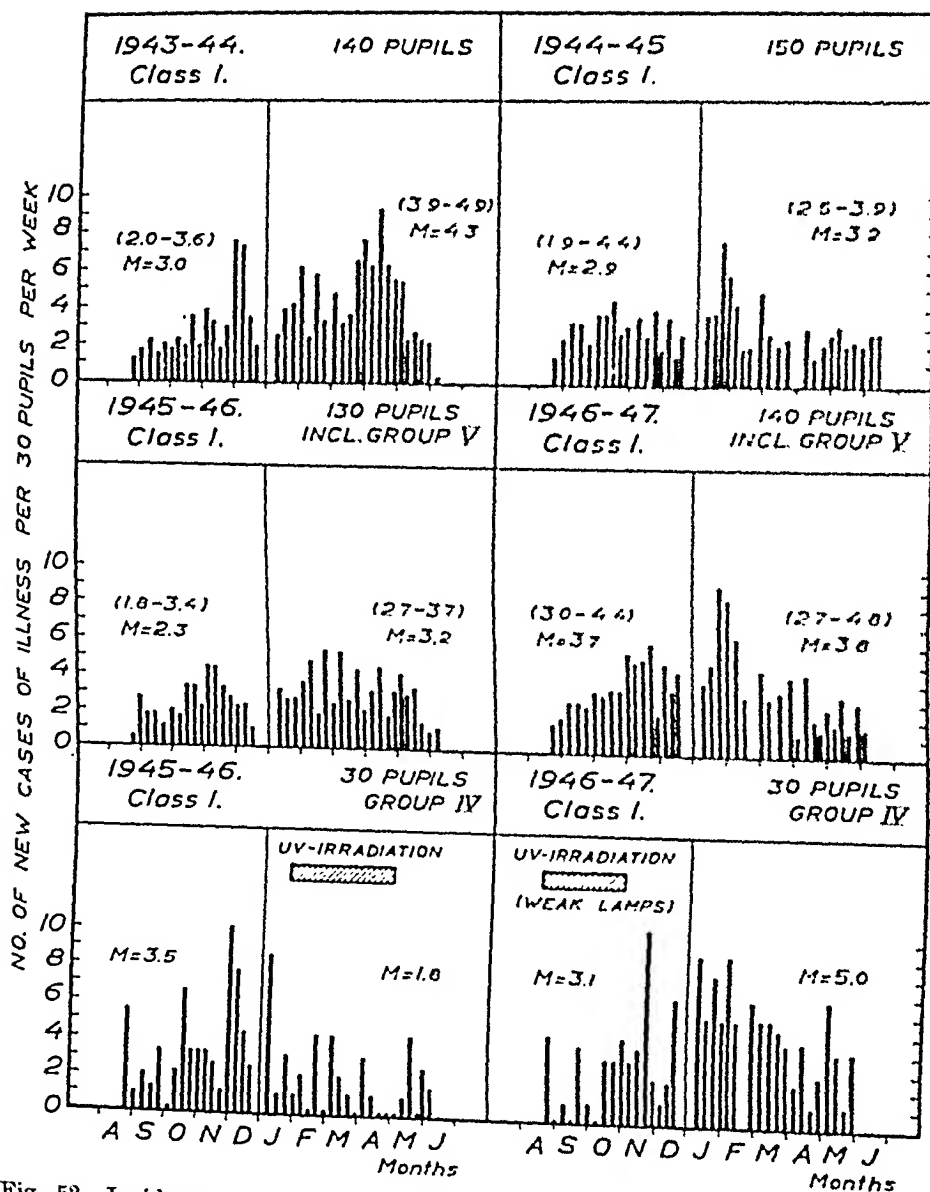


Fig. 52. Incidence of illness in 1st classes for each week of the school-years 1943-1947. Classes with UV-illumination in the two lowest squares (1945-1947).

dence of illness is given as the average number of new cases of illness per 30 pupils per week (6 days), and the time lost is expressed in school days per 30 pupils per (full) week. Every school year, comprising an autumn term between August and December and a spring term between January and June, is treated as an entity. The small figures placed in each term-square give the average for the term (M)

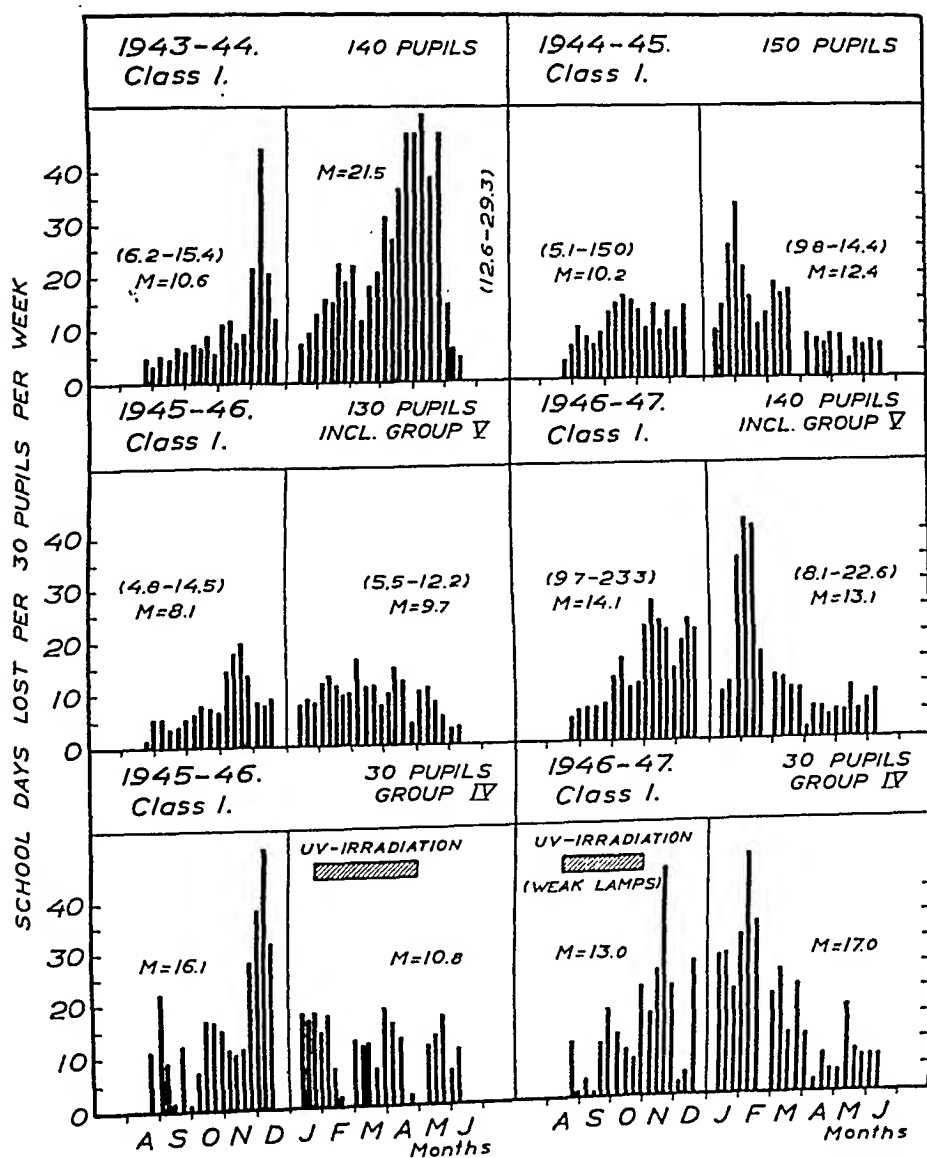


Fig. 53. Lost time due to illness in 1st classes for each week of the school years 1943—1947. Classes with UV-illumination in the two lowest squares (1945—1947).

and the variation range of the different classes (in brackets). The two lowest squares in every figure show the absenteeism recorded for the experimental classes of the investigation; the periods of UV-illumination are specially denoted.

There is a rather constant feature observed for the annual course of the absenteeism among the children, which appears in most of the

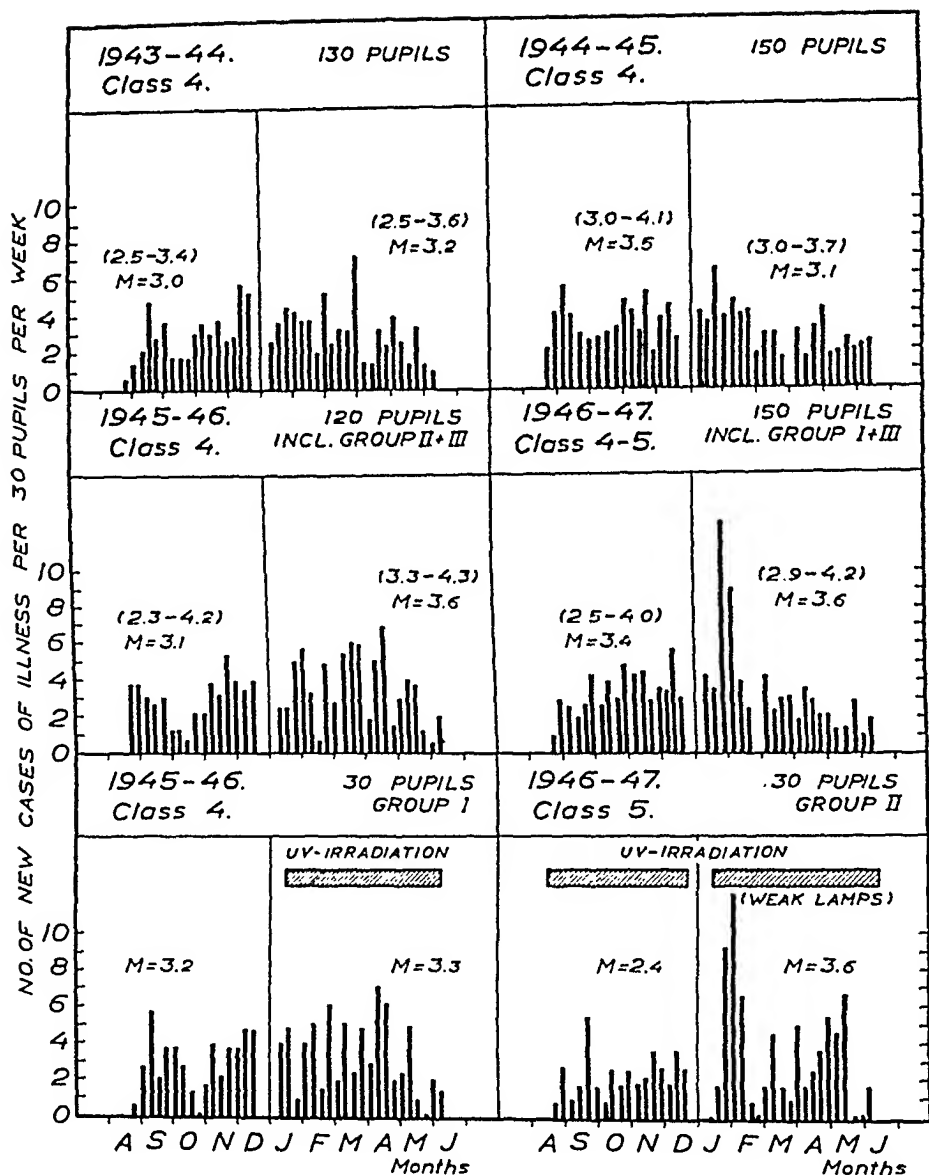


Fig. 54. Incidence of illness in 4th classes for each week of the school-years 1943-1947. Classes with UV-illumination in the two lowest squares (1945-1947).

diagrams and is particularly marked in the older classes. The first 4 to 6 weeks of the autumn term are characterized by a peak in illness, while the last weeks of October almost consistently show a pronounced minimum. From there the absenteeism increases again toward the Christmas holidays. Again, the first weeks of the spring term usually show a marked peak, which may rise to high values

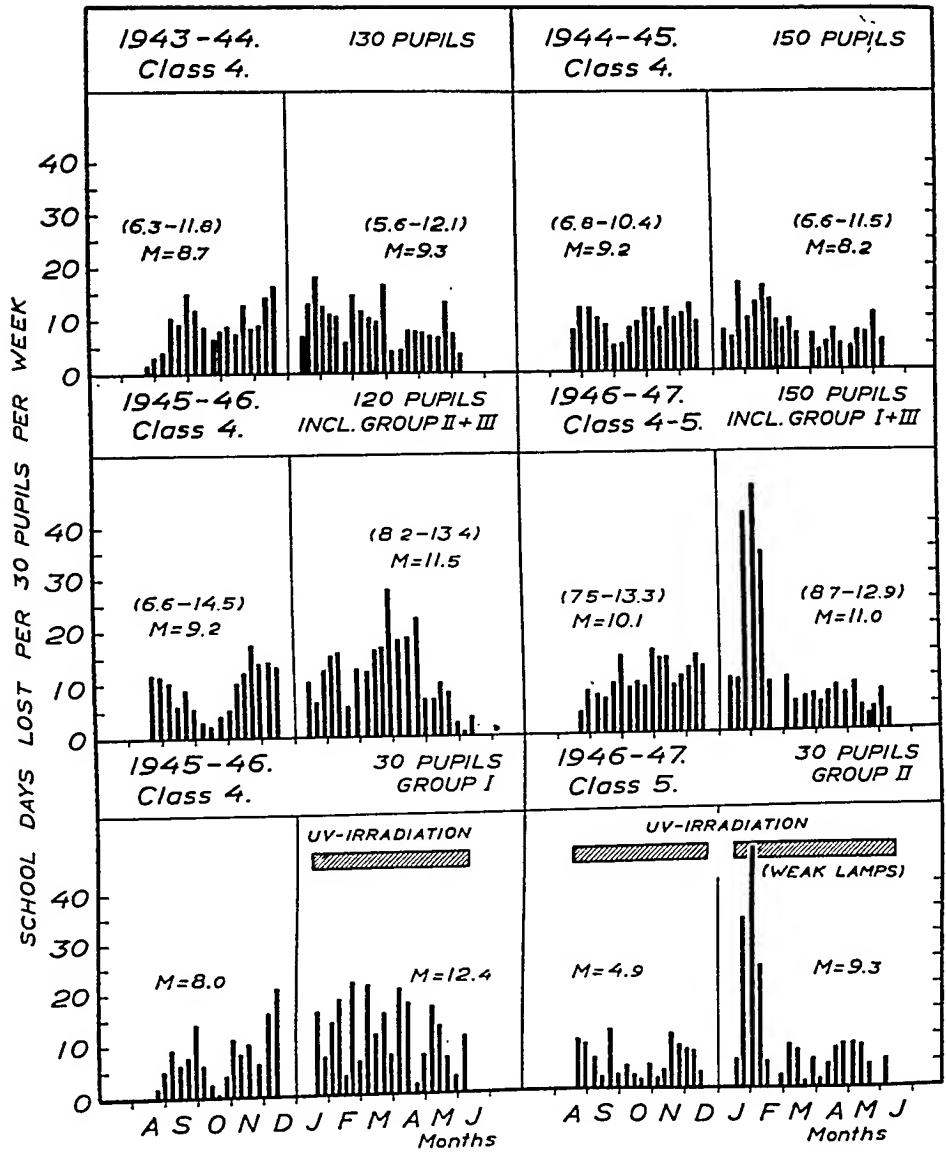


Fig. 55. Lost time due to illness in 4th classes for each week of the school-years 1943—1947. Classes with UV-illumination in the two lowest squares (1945—1947).

(see spring term 1947). A new maximum is often appearing about March, while the latest weeks of the spring term show lower absenteeism. It seems probable that the peaks appearing at the beginning of each term are due to the cross-infections occurring between the children when they are crowded together in the school. The average absenteeism is in most cases higher for the spring term than for the

autumn term. A similar general feature of the course of absenteeism from school is previously found by HJÄRNE (1942) on a comparable but smaller material from secondary schools in Uppsala.

The variations which may occur between different years are clearly demonstrated in, e. g., fig. 53. The time lost in spring 1944 is almost twice that in spring 1945 in these Primary school classes. This difference was less marked for the incidence of illness. Furthermore, the great variations which may occur between different classes of the same grade in the same term are illustrated by the small figures given in brackets for each term record. These give the range of variation of the averages for the different classes included in the total average, denoted by M . It is apparent that the deviation found for a single class from the corresponding average must be considerable before any significance can be attributed to it. A difference between different years is still more uncertain, at least for single classes. This must be considered in the interpretations of the results of UV-illumination.

During the spring term 1946, i. e., the first term of the experiment, there was no significant effect on the absenteeism among the older children in the Secondary school class (Group I) provided with UV-illumination (see figs 54 and 55). In the Primary school class (Group IV), on the other hand, the incidence of illness (fig. 52) was markedly low. *It was, in fact, lower than ever recorded for a 1st class in a spring term during the years 1943—1947.* Furthermore, the mean incidence during the spring term was only about half of the mean incidence of the autumn term. Such a relationship has not been seen in any other case. It seems, therefore, justified to assume that the UV-illumination really produced a decrease of the incidence of illness in this class for this period. As mentioned in a previous chapter, the intensity of the ultraviolet irradiation was particularly high in this classroom, which was made apparent by, among other things, the skin pigmentation developing in the children during this period.

The average number of school days lost during the same term (fig. 53) seems not to deviate from the average of the control classes. However, *a marked decrease occurred compared with the preceeding autumn term*, and this latter value was considerably higher in the experimental class during this autumn than the average of the control classes. This indicates that the duration of absenteeism for every incidence was longer in the experimental class, and that the effect of the

irradiation was to decrease the level to about the same as found in the control classes. The reason for the longer duration of absenteeism for each incident illness case in the experimental class may not necessarily indicate more severe illnesses; it may also be due to the parents keeping the child at home for a longer time for the same severity of illness, which, in turn, may be due to some differences in their social recruitment. This school is, as previously mentioned, situated in a residential part of the town with its, in general, higher social standard.

During the following autumn term 1946 the ultraviolet lamps in this class had decreased considerably in ultraviolet output, and it was impossible to have them exchanged. No significant effect of this weaker irradiation can be read from the diagrams.

Also the lamps of the Secondary school Group I had now become weak, but new lamps were installed in the classroom of Group II. *There is some evidence that the mean incidence and particularly the mean time lost was decreased by the irradiation in this class.* Group I, with weak lamps, is for this term included in the control groups; there was no indication of an effect of the irradiation.

During the spring term 1947 only the room of the Secondary Group II was equipped with effective UV-illumination, and the lamps had been burning during the previous autumn term. No significant effect may be concluded for this term. The inability of the irradiation to prevent the rapid spread of a respiratory epidemic appearing in January and February is clearly seen from the diagrams.

The results seem to indicate that the UV-illumination employed in the classrooms may to some extent decrease the absenteeism due to illness among the children *when the irradiation intensity is kept at a high level*, namely, that considered as the highest applicable for these cases in the design of the system. Such an intensity may be maintained without damage to the children; the depletion occurring is due to the unavoidable ageing of the UV-sources.

Discussion.

As is emphasized at the beginning of this chapter general conclusions regarding the effect of UV-illumination on sickness-rate in school children must be drawn with great care from this small ma-

terial. The results obtained do, however, provide a stimulus for investigations on a larger scale concerning this possible and favourable effect of the illumination.

The mechanism of such an action may *a priori* be explained in two quite different ways: the irradiation of the children may produce an *increased resistance to infections*, or, on the other hand, the irradiation of the air *depletes the air-borne spread of infections* in the classrooms and thus indirectly decreases the rate of illness. All the evidence is in favour of this latter theory. A marked decrease of the bacterial content of the air has been proved, and the bactericidal effect produced in the rooms is calculated to correspond to about that sanitary ventilation which is found to be effective for control of air-borne infections; this may be particularly valid for the high-intensity irradiations which seem to have influenced the absenteeism in this investigation. Periods of weak irradiation did not show significant effects in this respect while an influence directly on the children, as indicated by the mineral metabolism and physical fitness, were still apparent. It seems probable that the intensities of ultra-violet radiation in the classrooms necessary for preventing respiratory epidemics are considerably higher than those for exerting physiological effects. However, a possible influence on the resistance to infections in the children cannot be completely excluded from the present findings.

It should be emphasized that the air-disinfection of UV-illumination can influence only that spread of infection which is present in the classrooms. During the breaks, in the corridors, and out of school the children may be exposed to still stronger infection doses than in the classrooms, and the total depletion of respiratory infections can for that reason not be expected to be more than partial. The especially marked effect in the Primary school class during the first term of the investigation may to some extent depend on the absence of other classes in the building where this class resided.

Furthermore, the UV-illumination was kept burning only for some hours of the school days. In the 4th class during the spring term 1946 (irradiated Group I) the daily exposure was consistently maintained at 3 hours daily, while the illumination in the Group II during the autumn term 1946 was usually kept burning for longer times a day. This may be a reason why the absenteeism seems to have been more influenced in this latter group.

IV.

General Discussion.

The results obtained during the present investigation on ultraviolet irradiation of interiors in connection with the artificial illumination may be considered from different points of view. The technical section leads to some conclusions regarding the design and arrangement of an artificial illumination with that dual purpose. The physiological section deals with the effects of the irradiation directly on the occupants, in this case school children, and the hygienic section has been concerned with the bactericidal effects and the potentiality of reducing air-borne infections by means of this. For both of these aspects the results permit some conclusions of interest. However, the main question which arises from the investigation concerns the *general value and applicability* of such an illumination. This final decision involves as a further aspect the economic consequences. The possibility must be taken into account that the favourable effects which seem to be obtained with UV-illumination may also be reached with other more practicable and less expensive means. Thus, the ultraviolet irradiation of the children might be replaced by an adequate vitamin supply, and the bactericidal effect might be obtained with an installation of bactericidal low-pressure mercury lamps of considerably smaller total wattage than required for the UV-illumination in the present arrangement.

Leaving these aspects of economy out of the question for a moment the general conclusions obtainable from the present investigation may be summarized in the following way.

General Conclusions.

1. — The present state of knowledge with regard to spectral and dosimetric relations of ultraviolet radiation effects on man provides an acceptable basis for the establishment of the fundamental re-

quirements of the spectral characteristics of the ultraviolet source to be employed for general use in illumination. The knowledge is also sufficient for an estimate of the dimensional design of each installation which must be made with consideration for the time of exposure to the illumination. *The ultraviolet irradiation component may be treated as a separate entity in the design of the system, apart from the illumination component, according to the special photochemical laws valid for biological irradiation effects.*

2. — The ultraviolet radiation may suitably be *resolved into different action components* on the basis of different action spectra; the principle is the same as the resolution of light into different colours. The responses to be separated are the pigmentation effect, the antirachitic effect, the erythema effect, and the ophthalmia effect, all of which concern the direct action on the occupants, and the bactericidal effect, which concerns the action on air and environment. It seems probable that for practical purposes the erythema effect and the antirachitic effect can be referred to the same action spectrum, as well as the photophthalmia effect and the bactericidal effect.

3. — The interpretation of available data on action spectra and threshold exposures has shown that for the direct irradiation of occupants the *short wavelength limit of the radiation* ought to be situated at about 290 m μ . No favourable effects are gained by radiation of shorter wavelengths which are not obtained with greater effectivity and safety by the longer wavelength range. This limit is found in the solar radiation during optimal conditions. In such a radiation the specific erythema component and the antirachitic component, both limited to the range below 315 m μ , may be treated together, i. e., with the same units, and the erythema effect becomes a reliable measure of the antirachitic effect. The risk of photophthalmia is avoided in exposures up to the normal erythema threshold level.

However, the *bactericidal effect* which provides a highly desired action of the illumination is mainly dependent on the prevalence of radiation of wavelengths shorter than 290 m μ . The addition of these wavelengths to the illumination increases the ophthalmia risk in about the same proportion as it increases the bactericidal effectivity.

4. — The investigation has shown that *a compromise is possible, with a resultant illumination of marked bactericidal action without obviously injurious and unfavourable effects.* The displacement of the

spectral intensities from the extreme short wavelength of the specially designed bactericidal low-pressure mercury lamp towards the medium range 260—315 m μ permits the uniform distribution of the radiation in the room. The bactericidal effect obtainable by this in the room is sufficient for the maintenance of a considerable sanitary ventilation potential for reducing air-borne spread of infections.

5. — The basis for the dosimetric design of the irradiation component of the illumination was made by the average normal threshold erythema response. It is shown that *daily exposures to that level of the face and hands of children secure marked effects on mineral metabolism and physical fitness without causing any obvious discomfort*. It seems probable that the same benefits may also be reached with smaller daily exposures, but the higher exposures provide at the same time a stronger bactericidal effect.

6. — One way for the technical arrangement of such an artificial illumination with the dual purpose of providing a simultaneous illumination and irradiation of the interiors is shown. The *medium-pressure mercury vapour lamp* with the outer bulb of special ultraviolet-transmitting glass can be combined with incandescent lamps in such a proportion that a good illumination with pleasant colour of the light is obtained at the same time as both the visible and ultraviolet radiant intensities are suitable for their respective purposes. The total illumination level necessary to provide a pleasant illumination for an erythema-effective exposure is very reasonable. *To produce in about three hours an erythema threshold response in untanned human skin the illumination level required for a pleasant colour of the light is about 300 lux*. This level may be decreased if longer exposures are available. By having the ultraviolet source separated from the main sources of illumination a great range of variation between illumination and irradiation is possible to establish. The source may easily be made so that it radiates a suitable proportion of short-wave, bactericidal radiation.

7. — The technical features of the installation of UV-illumination include the trinity of *source, reflector, and paint*. The reflector should prevent glare and procure a uniform distribution of the radiation, both visible and ultraviolet, for which special assumptions are necessary. Ultraviolet-reflecting paints on ceilings and walls constitute an important factor for the final result with regard to uniformity of irradiation and utilization of the ultraviolet radiation from source

and reflector. These principles are independent of the type of lamp used as source for the ultraviolet radiation.

8. — The *physiological effects* on school children of daily exposures to such an illumination during the winter are found to be very marked in two respects: *mineral metabolism* and *physical performance (fitness)*. The effects are in the main acting toward a *counter-balance of seasonal variations of these functions*, recorded for the untreated children. These seasonal variations follow an annual course theoretically expected for functions which are subject to a dominant influence of substances photochemically formed and stored in the body by the solar radiation. The optimal phase, characterized by blood-chemically evaluated ideal conditions for skeletal growth, and work-test evaluated high scores of physical fitness, are found in the autumn, around the equinox, when the accumulation of solar-formed substances should be a maximum. The negative phase, with the reverse characteristics, appears for both functions in the spring, around this equinox, when the store of solar substances should have its minimum. This seasonal course is completely cancelled by the ultraviolet irradiation during the winter. *A definite proof is obtained hereby of the important role normally played by the direct action of solar radiation on children during the summer half-year for both of these functions.* The consequences of the poverty of ultraviolet energy in the outdoor daylight during the winter are also apparent from this, which emphasizes the desirability of some form of substitute especially for the countries situated in the north.

It should be pointed out that a high degree of *pigmentation* cannot be expected to develop from exposures to this illumination. The pigmentation effect is mainly limited to the spectral range 315—400 m μ , where the mercury arc lamp radiates only weak intensities, and the dosages necessary for an effect in this respect are too high for convenient application by an illumination system even if other sources are used.

9. — The *mechanism of action* of the radiation must be found for both functions in the action of some photochemically formed substances. It has not been the purpose of the present investigation to find them, but there are several reasons collected which support the view of a *vitamin D mechanism as the primary action*. The possibility of an influence of vitamin D or related substances on muscle metabolism and working capacity provides a great stimulus to further

research. In any case, it is shown that normal unsupplemented winter diet of children is not a sufficient source for these substances and that in this meaning *children suffer from some degree of "solar vitamin" deficiency during the winter and especially the early spring.*

10. — The hygienic effects of the UV-illumination are concerned with its *bactericidal properties* and the possibility of reducing air-borne spread of infections. The contradictory requirements on spectral quality for disinfection efficiency and physiological efficiency placed the bactericidal potentiality of the UV-illumination as a secondary factor in the initial design of it. The investigation showed, however, the inherent possibility in that respect, and it is obvious that *this effect must be treated as a factor of primary importance.* The UV-illumination offers the first artificial illumination system with marked bactericidal effect, and it seems probable that it will find some fields of application for this purpose as a complement and sometimes a substitute for the specially designed bactericidal lamps. *Low-ceiling rooms and rooms with poor air-circulation* are previously mentioned as suitable applications.

Further experiments on this line are going on i day-nurseries, and a new reflector which permits an intense horizontal distribution of the radiation is constructed in order to utilize the bactericidal power to a higher extent than was made in the arrangement used for the school experiments reported here. In this way a high-intense radiation barrier is laid just above eye-level in the room, which produces an effective sterilization of the circulating air at the same time as the illumination below eye-level maintains a considerable bactericidal efficiency.

Practical Conclusions.

Returning to the practical question from the public health point of view regarding the justification of a recommendation of UV-illumination for general use, it is seen that the price of the illumination is outweighed by several distinct advantages. A good artificial lighting is obtained at the same time as favourable physiological effects are produced by the direct action of the radiation on the occupants, and a potential reduction of infection-spread results from the action of the radiation on the micro-organisms of the air. The only disadvantages concern the *high wattage* required for it (about 40 watts per sq. m. floor area) and to some extent the comparatively

high price of the ultraviolet source. The cost of the installation of UV-illumination in, for instance, an ordinary classroom is, however, rather smaller than the cost of an installation of the special bactericidal lamps for upper-air irradiation. The additional cost of installing an artificial illumination of the level reached by the UV-illumination is of about the same magnitude. The high wattage of UV-illumination in the present state is mainly due to the incandescent lamps which provide for the pleasant illumination. For use in schools and similar localities the power may be reduced by keeping only the ultraviolet sources burning during the bright hours of the day. It is found that the colour of the mercury light is then almost completely merged into the daylight. The power of this irradiation is about 10 watts per sq. m. floor area, compared with about 2 watts per sq. m. for upper-air irradiation with low-pressure mercury bactericidal lamps. For this higher price of the UV-illumination the direct physiological effects on the occupants are gained.

The wattage for the illumination component may be greatly reduced by using fluorescent lamps instead of incandescent lamps. With such lamps of comparatively low colour temperature ("white" or "soft-white" types) it seems possible to get a resultant illumination of suitable colour.

Finally, the principle of UV-illumination must be considered with regard to the use of other ultraviolet sources which may be on the market in the near future. The fluorescent ultraviolet lamp is still in the experimental state but appears to be an efficient and suitable source of ultraviolet combinable with fluorescent illumination. The high-pressure mercury vapour lamp emits radiation of a spectral composition which seems to be well adapted for the same purpose (see fig. 21, p. 89). It is still impossible to predict the bactericidal effect obtainable by these sources.

It seems probable that some of the physiological effects of UV-illumination may be reached by the simple administration of sufficient doses of vitamin D to the children during the winter. From the economic point of view this would obviously have distinct advantage over the UV-illumination. The final decision must, however, be postponed until further investigations are made on this fundamental question.

The natural field for the application of UV-illumination would tentatively be *schools, day nurseries, and similar localities where*

children are brought together especially during winter months. In hospital wards with the patients having their faces and eyes directly exposed to the radiation from above, there may be risk of harmful effects if such high intensities are used as in the present experiments. The utilization of the illumination for homes is still limited by the cost and the wattage. The damaging effect on flowers and plants may be a further disadvantage for the home application. The control of over-exposure cannot be provided in the same degree as in public localities.

The use of UV-illumination in *localities for adults*, as in industry, under-ground factories, etc., is probably justified mainly by the bactericidal properties of it. The growing organism may be expected to be more sensitive to the deficiency of biologically-active radiation during the winter than the adult organism, and the desirability of a supplementary ultraviolet irradiation of adults cannot be stated with the same certainty as in the case of children. It should be pointed out that the average industrial worker receives almost no ultraviolet radiation for the most part of the year. He is exposed to outdoor daylight only in the morning and afternoon hours when practically no ultraviolet radiation reaches the earth, and no such radiation penetrates to indoor working places. There are obviously no apparent harmful effects developing from this, which, however, would probably be the case for children. Workers in under-ground factories, miners, etc., are not subject to worse conditions than the average industrial worker in this respect of short-wave ultraviolet rays. It may, however, be possible that the deprivation of ultraviolet radiation does manifest itself by some degree of "spring fatigue" also in adults. Tests on working efficiency as applied here on children would provide one means of deciding this.

The bactericidal purpose alone may in most industrial localities be reached in a more economical way by means of the special low-pressure mercury bactericidal lamps.

Summary.

I. Introduction.

Chap. 1. *The Basis and Purpose of the Investigation.*

The purpose of the investigation was to study the physiological and hygienic effects of the addition of biologically-effective ultraviolet radiation to the general illumination of interiors. It is emphasized that from the physiological point of view this problem is of special interest to countries situated in the north where the natural supply of this radiation is poor for long times of the year.

Chap. 2. *A Brief Survey of Ultraviolet Radiology.*

A brief review is given of ultraviolet radiology with special reference to sources of ultraviolet for biological purposes, and the biological effects on man of ultraviolet radiation. Besides the local effects, such as erythema, pigmentation and photophthalmia, irradiation is proved to be a potential factor for mineral metabolism and for muscular working metabolism.

Chap. 3. *Hygienic Applications of Ultraviolet Radiation.*

Previous attempts to provide for ultraviolet radiation in interior localities by means of ultraviolet-transmitting window glass and artificial sources are reviewed; the former is mostly inefficient, the latter is not yet adequately studied. Results obtained in large-scale investigations on the benefits of artificial sunlight treatments in the usual way with 'solaria' have given no evidence of decreased sickness-incidence or duration. Physiologically estimable increases of muscular working capacity may, however, result. A modern hygienic application of ultraviolet radiation is made by the radiant air disinfection for control of air-borne spread of infections. An efficient source for this is the bactericidal low-pressure mercury arc.

II. *The UV-Illumination System.*

Chap. 4. *The Basic Design of the UV-Illumination System.*

The design of the UV-illumination system was made with careful consideration of spectral and dosage relations of ultraviolet irradiation effects on man, of which a detailed account is given. The importance of the time-factor is stressed. A system of terms and units intended for practical purposes is described. It was shown that for the action on the occupants the short-wave spectral limit of the radiation ought to be situated at the wavelengths 280—290 m μ , although the prevalence of shorter wavelengths is accompanied by the benefits of a stronger bactericidal effect on air and environments. The system was designed for classrooms and so that the daily exposure should approximately reach the erythema threshold dosage on the face and hands of the children.

Chap. 5. *The Technical Arrangement of the UV-Illumination System.*

The technical arrangement comprised of radiating source, diffusing reflector, and reflecting paints on ceilings and walls. The source consisted of a medium-pressure quartz mercury-arc enclosed in an outer bulb of special glass permitting the emission of radiation in the medium-wave range 280—315 m μ but mostly absorbing radiation of shorter wavelengths. A pleasant colour of the illumination was obtained by supplementary incandescent lamps. A type of reflector, and some ultraviolet-reflecting paints, developed during the investigation, are described. The total wattage required for the illumination of an ordinary classroom of 50 sq. m. floor area amounted to about 2 kilowatts, most of which was due to the incandescent lamps.

Chap. 6. *The Radiation Climate of the Experimental Rooms.*

The intensities of ultraviolet radiation obtained in the classrooms equipped with UV-illumination were computed and experimentally controlled to be at the level required for erythema-effective exposure in 3—5 hours. In the more intensively irradiated room the children developed a slight but visible degree of pigmentation. No single case of harmful effects occurred in spite of the comparatively high intensity of short-wave ultraviolet radiation.

Chap. 7. *The Physical Methods Employed.*

The physical methods employed for measurements of spectral energy distribution, spectral transmittance and reflectance and spectral radiant intensities are described. A new semi-quantitative spectrographic method for the direct estimation of spectral energy distribution is developed.

Chap. 8. *A Comparison with Daylight Ultraviolet Conditions.*

Data available on the spectral energy distribution in sun and sky radiation at different elevations of the sun were used to give a basis for comparison with the artificially created indoor ultraviolet radiation climate. The daily amounts of antirachitic radiation were computed for different seasons and different latitudes. Practically no such radiation reaches the earth during 4—5 of the winter months at the latitudes of Scandinavia. The maximal annual amounts, calculated for a presumed cloudless year, increase markedly towards southern latitudes. The artificially made radiation climate offers some resemblance to the natural outdoor radiation conditions at a sun elevation of 30 degrees with respect to antirachitic and erythema effect but is much weaker in pigmentation effect.

III. The UV-Illumination Effects.

Chap. 9. *A General Scheme of the Investigation.*

The investigation of physiological and hygienic effects of the UV-illumination was carried out in a Primary and a Secondary school of Uppsala during a period of 1½ years. A total of 220 children aged about 7 and 10 years, respectively, were involved in the investigation, 120 of whom received ultraviolet irradiation for some period of time. The methods of the statistical treatments and the reproduction of the results are described.

Chap. 10. *Effects on Some Constituents of Blood.*

Blood analyses on the children were carried out with regard to the levels of haemoglobin, total calcium, inorganic phosphorus and alkaline phosphatase. Six series of determinations were made, distributed over different seasons of the year. A seasonal variation in

untreated groups was recorded for the plasma level of calcium, with a maximum concentration in September or October and a minimum concentration in March, and for the plasma phosphatase activity, with the seasonal course reversed to that of calcium. In the irradiated groups these seasonal variations were completely counterbalanced, and the concentrations maintained at the normal autumn levels during the winter. The levels of inorganic phosphorus as well as of haemoglobin showed no significant variations with the seasons, and no pronounced effects were obtained by the ultraviolet irradiation.

The administration of a single dose of 250,000 I. U. of vitamin D (calciferol) to a small group of non-irradiated children in mid-winter did not significantly influence either of these blood levels.

The interpretation reveals the dominant and selective importance of ultraviolet radiation for the maintenance of the mineral metabolism at the optimal level found in autumn in these school children. The seasonal variations are explained as due to the state of accumulation of solar-formed substances in the body; the normal winter-diet is not sufficient to replace the lack of natural ultraviolet radiation. The mechanism of action of the radiation, whether solely due to formation of vitamin D or if a specific hypercalcaemic effect is also involved, is discussed.

Chap. 11. *Effect on Physical Fitness.*

The scores of physical fitness were obtained for Secondary school boys in irradiated and control classes (60—90 boys) in six series of determinations applied at different seasons. The test comprised a set of cardiovascular criteria applied in the submaximal phase of a continuously graded work on a bicycle ergometer. Detailed accounts of the method and the experimental controls of reliability carried out are given. Untreated control children showed a tendency to a seasonal variation in physical fitness, with the highest scores in autumn and lowest scores in spring. By means of the UV-illumination the scores were maintained or rather increased during the winter, and the difference between irradiated and control children was especially marked in the spring.

The vitamin D dosage administered to the control group strongly indicated an effect in the same direction as obtained by the irradiation.

The results emphasize the importance of solar ultraviolet radiation and its accumulation in the body, probably in the form of vitamin D, as a factor for the general well-being of children. Decrease of working capacity seems to constitute a sensitive sign of vitamin D deficiency. The findings are discussed in the possible relation to "spring-fatigue."

Chap. 12. *Effect on Air-Borne Bacteria.*

The bactericidal effect of the UV-illumination was studied on a laboratory scale by infection-chamber experiments, and by counts of total bacterial concentration of the air in the experimental and control classrooms. A physical interpretation of radiant air disinfection is made with special reference to the uniform irradiation of the room obtained with UV-illumination. Theoretical as well as experimental evidence is given for a killing-rate of respiratory air-borne organisms in the zone of the occupants corresponding to an equivalent ventilation of about 30 air-changes per hour. The average count of total bacteria in irradiated classrooms was found to be about half the count in control classrooms; the reduction of most pathogenic bacteria may be expected to have been still greater.

Chap. 13. *Effect on Absenteeism.*

Records of absenteeism due to illness were made for each week of the terms for different groups of school children in Uppsala for the years 1943—1947. The features of the seasonal course are discussed. On the basis of these statistics it is shown that the UV-illumination in some cases caused a marked reduction of absenteeism; the effect in this respect seemed to depend on the intensity of the irradiation and ceased when the ultraviolet sources began to age. The effect is ascribed to the air-disinfection obtained by the use of the UV-illumination.

IV. General Discussion.

The results are discussed with respect to the general conclusions regarding technical, physiological, and hygienic aspects of the UV-illumination which are emerging from the investigations. An application of this kind of illumination seems recommendable especially in localities as schools and day-nurseries where children are brought together. The bactericidal effect, particularly, may justify its use in other cases also.

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ACTA PHYSIOLOGICA SCANDINAVICA

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POTASSIUM AND THE DIFFERENTIAL
THERMOSENSITIVITY OF MEMBRANE
POTENTIAL, SPIKE AND NEGATIVE
AFTERPOTENTIAL
IN MAMMALIAN A AND
C FIBRES

by

ANDERS LUNDBERG

Stockholm 1948



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Preface

The present work has been carried out at the Nobel Institute for Neurophysiology. When in 1946 I was attached to this laboratory I became engaged in work on selective thermostimulation of different types of fibres and out of this engagement arose the theme of this paper.

It is a pleasure to record my gratitude to Professor Ragnar Granit for advice and criticism and for his stimulating interest in my work.

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Stockholm, March, 1948.

ANDERS LUNDBERG

Introduction and problem

The work to be described in this paper contains an analysis of the thermal properties of mammalian A and C fibres. Selective differences in thermosensitivity had been described in this laboratory for various nerves. Since these papers served as a starting point for my own work I will first give a brief summary of their content.

BERNHARD and GRANIT (1945) took up thermostimulation of nerve in order to study a simple model temperature end organ. They found that impulses were discharged when the nerve was heated or cooled from a zero temperature of 37° . In both cases the locally heated or cooled region became electronegative relative to nerve at their equilibrium temperature of 37° . They assumed that the heat or cold potentials thus created served as generator potentials initiating the discharge of impulses.

These experiments were further developed by C. v. EULER (1947) and by GRANIT and LUNDBERG (1947). They proved that local cooling of mammalian nerves only stimulated the large myelinated fibres, whereas local heating to 45° in an equally selective fashion stimulated the thin afferent fibres. An extensive analysis of these effects by C. v Euler demonstrated that heating stimulated small afferents including the C group.

v. Euler also took up the question as to whether the heat and cold potentials arose in fibres of different diameter. By choosing peripheral nerves, as homogeneous as possible with respect to fibre size, he arrived at the following conclusions: (i) local heating from 37° to 45° renders the heated region electro-negative in nonmyelinated and in thinly myelinated fibres of the B group whereas no changes whatever or very small ones occur in large myelinated fibres; (ii) on the other hand, the large myelinated nerve fibres remain practically uninfluenced by heating but respond to local cooling from 37° downwards with the characteristic cold potential, mentioned above.

By thermostimulation and an analysis of the changes in the membrane potential introduced by local cooling and warming of nerves it has thus been demonstrated that there are differences in the thermal sensitivity of various fibre types. Since it is probable that such dissimilarities are due to important differences in the physico-chemical properties of the structures concerned a systematic analysis of the effects of temperature changes was undertaken.

In this work A and C fibres have been studied separately and their properties compared. Characteristic differences have been found for the two fibre groups. The membrane potential, the spike and the negative afterpotential are features of nerve activity which have been systematically studied in relation to temperature changes. These results have been described in Part I. Part II is devoted to an analysis of the effects of potassium on the thermal sensitivity of membrane potential, spike and negative afterpotential by means of variations in the concentration of potassium ions in the external medium surrounding the nerve. The A and C fibres were found to be differentially sensitive to temperature changes when investigated by means of the functions enlisted above as indicators. It was possible to establish different temperature optima in the two kinds of fibres with respect to those indicators. Modest variations in the concentration of potassium ions were found to shift these optima so that, within limits, it became possible to change the specific thermal properties of the A fibres in the direction of those of C fibres or *vice versa*. Thermoanalysis of the two fibre groups thus has provided a system of correlations as well as a method of approach to be described in detail below.

PART I

The thermal sensitivity of membrane potential, spike height and negative after-potential in mammalian A and C fibres

Historical section

In the last century interest in the effects of temperature upon nervous tissue was centred around questions concerning nerve irritability, thermal stimulation and the cold block. But with the advent of the membrane theory it appeared that subjecting the nerve to temperature changes offered possibilities for establishing the nature of the membrane. BERNSTEIN (1902) experimented along such lines. HERMANN (1871) showed that a cooled portion of a muscle became electronegative relative to a region at normal temperature and GRÜTZNER (1881) confirmed this observation for peripheral nerve. VERZAR (1911) returned to this aspect of the problem and confirmed Grützner's observation. Using frog nerve he also noted that heating above 20° reduced the membrane potential. The effect was described as the setting up of a demarcation potential. Actually, what he observed, was that the membrane potential of frog nerve has its maximum around 20° . The depolarization due to cooling was small, only around 1 mV. Similar values were obtained by BREMER and TITECA (1934) who, however, found the depolarization due to heating to be of a larger order.

As already pointed out above, BERNHARD and GRANIT (1946) used mammalian nerve and the body temperature as their equilibrium temperature. Starting from this level both heating and cooling caused depolarization. C. VON EULER (1947) came to the conclusion that the "cold potential" was set up in large

myelinated fibres, the "heat potential" in thin fibres (see above, Introduction).

Several authors have described effects of temperature upon the action potential. GASSER (1930) and AMBERSON, PARPART and SANDERS (1931), demonstrated that the negative afterpotential in A fibres (frog) was small at low temperature. The effect of cooling upon the spike was studied on frog nerve by GASSER (1931). The animals were acclimatized to 30° and, when the nerve was cooled starting from this temperature, the spike diminished and disappeared at about 5° . At the same temperature the nerve became inexcitable. Gasser also found that the durations of the rising and falling phases of the spike were symmetrically extended by cooling. As to the mechanism underlying the diminution of spike height at low temperatures he indicated, basing himself on a comparison with the results of Verzar, that the spike potential could not be determined by the resting potential. In connexion with their work on the influence of temperature upon the membrane potential BREMER and TITECA (1934) observed that in frog nerve spike height was maximal at $15-22^{\circ}$, *i.e.* the temperature to which their animals were acclimatized. SCHOEPFLE and ERLANGER (1941) studied the effect of cold on their single fibre preparation in the frog's toe nerve. Cooling to 8° augmented the spike height and, contrary to Gasser, they found that the descent of the spike was far more prolonged by cooling than its ascending phase. The temperature to which their frogs were acclimatized is not given.

An extensive analysis of the effect of temperature on the action potential of frog nerve has been carried out by LORENTE DE NÓ (1947). The changes in the action potential brought about by cooling were identical with those produced by lack of oxygen or by depolarizing agents. In his experiments the spike had its maximum at $10-15^{\circ}$. Cooling was found to reduce his L-fraction of the membrane potential. At 6° it was a great deal smaller than at 18° and at 2.5° it was practically non-existent. By studying what he calls the R_2 deflection he also found that his M-fraction of the membrane potential diminished during

cooling so that, at a temperature of 0—2.5°, the membrane potential consisted only of his Q-fraction.

Lorente de Nó also discusses the question as to how the changes in the membrane potential, due to cooling, are related to the diminution of spike height and the final inexcitability. Having noted that cooling changes the spike in the same manner as anoxia and certain metabolic inhibitors which reduce the membrane potential he proceeds to demonstrate that the onset of inexcitability in the cooled nerve cannot be due to depolarization, even though, in general, a large enough reduction of the membrane potential is accompanied by inexcitability. Apparently the mechanism of spike generation is disturbed by the lowering of the temperature. The complexity of the problem is illustrated by the fact that externally applied anodal currents restore the excitability of the cooled nerve as if the loss of excitability really had been due to depolarization.

The many attempts to block impulse activity by temperature changes provide a great deal of interesting information. Thus it is an old observation that nerves of coldblooded animals are blocked by warming at lower temperatures than those of mammals, and it is also known that cooling blocks mammalian nerves at higher temperatures than frog nerves. The latter are very resistant to cold. The block occurs at —2° to —7° (BOYCOTT, 1902, BÜHLER, 1905, BAHRMAN, 1932 and BOYD and ETS, 1933, 1934). An interesting exception has been reported by GARTEN and SULZE (1913). They studied a tropic frog and found that the block occurred at a very much higher temperature, generally around + 5°, sometimes at still higher temperatures. Previously HOWELL, BUDGET and LEONARD (1894) had found that nerves from spring frogs also were blocked at relatively high temperatures, generally at +5°, but often at still higher temperatures.

Normally frog nerves are very sensitive to warming. THÖRNER (1920, 1922) observed with *Temporarias* that the blocking temperature was at 33°. THÖRNER (1922) and BREMER and TITECA (1946) have studied the mechanism of the block due

to warming. ADENSAMER (1934) found that higher temperatures were required for blocking frog nerves the further south the animals came from.

Far less cooling is necessary in order to block mammalian nerves. GRÜTZNER (1878), working with dogs, found the motor fibres in the sciatic nerves to be blocked at 6° whereas the sensory ones conducted down to 1° . In order to block the vagus BOYCOTT (1902) had to reduce the temperature to 0° . There is, however, some variation in the figures given by different observers. The experimental animals may also differ in thermosensitivity. Thus HOWELL, BUDGET and LEONARD (1894) reported that the vagus in dogs was blocked at 0° , in rabbits already at 15° .

Technique and procedure

In the experiments on A fibres the 6th and 7th lumbar roots of the cat were used. These nerves do not contain C fibres and are long enough for our purpose.

The cats were decerebrated in ether anaesthesia. After a one hour period of recovery the roots were removed as carefully as possible and placed into a bath containing Krebs solution.

For work on C fibres the splenic nerve of the cow was the standard preparation. This nerve was excised with appending vessels about 5 minutes after the slaughter of the animal and immediately placed into warm Ringer saturated with 93.7 % O_2 and 6.3 % CO_2 . They were brought to the laboratory and 15 minutes later dissected. This lasted about half an hour. They were then placed into the bath containing the Krebs solution, saturated with the same gas mixture as above, and left there to equilibrate for 2—3 hours before the experiments were begun.

Some experiments were also carried out with the roots of the cow. These could not be removed immediately because of the extensive operative procedures and so did not reach the bath immediately. On the other hand time was gained in the later phases because dissection in the laboratory only required a few

minutes. Afterwards the roots were kept in the bath for 4—5 hours before experimentation.

The nerve bath consisted of an outer case containing paraffin oil, heated electrically. A bowl in the liquid paraffin was the nerve bath proper and held about 400 ml saline. The nerve was subjected to local temperature changes with the aid of a 1 cm silver thermode containing a groove for the nerve. This thermode had been lacquered repeatedly with a special lacquer, each time dried at 300° in order to secure perfect isolation. The temperature of the thermode was regulated by a circulating fluid, in the beginning water but later paraffin oil. The temperature was controlled at the affluent branch of the thermode by means of a thermocouple connected to a Cambridge spot galvanometer for visual control. In addition it was recorded photographically by a mirror galvanometer critically damped to a period of one second. In order to preserve a reasonable amount of sensitivity without increase of film width, the galvanometer mirror was illuminated by two adjacent light beams in such a manner that when a large deflexion of the mirror removed one of the light spots from the film the other one took over and continued the record. A Jacquet clock interrupted one of the light beams for time marking.

Ag-AgCl electrodes were used for the recording of all potential changes described in this work. The nerve was placed in the thermode with one end dipping into the bath. One electrode was on the nerve in the thermode, the other one in the bath which also was earthed by means of a third electrode. The recording electrodes were taken to a directly coupled amplifier with a high input resistance operating a moving coil galvanometer critically damped to a period of one second. Action potentials were recorded in the customary way with one electrode on the severed end of the nerve and another one not less than 1.5 cm further down. The latter touched that part of the nerve that was inside the thermode.

KCl was not normally used in order to eliminate the diphasic artefact. The thermosensitivity of the nerve is very much in-

fluenced by an excess of potassium ions (*vide infra*). For this reason a diphasic artefact was held to be a minor evil than a suspected diffusion of potassium ions into the thermode region of the nerve.

The amplifier was condenser coupled with two alternative time constants of respectively one and ten seconds. The latter was employed for work on afterpotentials of C fibres. The shorter time constant was used in all other experiments.

For stimulation a device was available delivering rectangular pulses of variable intensity and duration. The shock artefact was controlled by a compensation bridge.

All experiments were performed with nerve or roots in Krebs solution; NaCl (0,154 M) 100, KCl (0,154 M) 4, CaCl₂ (0,11 M) 3, KH₂PO₄ (0,154 M) 1, MgSO₄ · 7H₂O (0,154 M) 1, NaHCO₃ (0,154 M) 21 volume parts; 1 g glucose per liter solution. The fluid was aerated continuously with the gas mixture containing 93.7% O₂ and 6.3% CO₂. This gave it a pH of 7.35. The gas was pre-warmed and saturated with humidity. The gas flow was regulated to about 1 liter per minute.

Artefacts. Analysis of the thermopotentials by the method outlined above is fraught with risks for artefacts. In the beginning the most troublesome artefacts were those introduced by the water passing through the thermode. Apparently the thermode served as a condenser picking up the potentials from an element formed by two different metals within the circulating system. These difficulties could be wholly eliminated by the use of paraffin oil as the circulating medium. It was also noted that artefacts occurred in the shape of thermopotentials when the resistance between the recording electrodes was too high. This can be observed with particularly thin nerves. But in a nerve of greater thickness the layer of humidity causes a reduction of interelectrode resistance which is sufficient to remove potentials due to differences in temperature between the two electrodes. Other artefacts observed and avoided by various procedures were of the kind seen in all electric recording and not characteristic for this work.

Results

Chapter 1. Variations in the membrane potential of A and C fibres in response to local temperature changes

It has been my aim in this investigation to study the thermal sensitivity of the membrane potential in nerves containing as much as possible of but one type of fibre (Cf. C. v. EULER, 1947) and for this reason the lumbar motor roots 6 and 7 were chosen as representatives for A fibres. These are lacking C fibres but possess in addition to the alpha fibres a group of small fibres (see ECCLES and SHERRINGTON, 1930, LEKSELL 1945). The roots have the further great advantage of being without perineural sheaths. The splenic nerve of the cow proved to be an excellent preparation for pure C fibres (studied from other points of view by Prof. U. S. v. Euler in the Physiological Department to whom I am indebted for having drawn my attention to this preparation). The experiments were carried out on nerves from which the perineural sheath had been removed, a procedure easy to perform. Controls showed that the "unsheathed" nerves did not differ in their temperature sensitivity from normal ones if they had been carefully handled during this operation.

A fibres. The thermopotentials recorded from excised nerves are somewhat smaller than those obtained from nerves *in situ*. The reasons for this difference were not examined by experimentation. It is possible that the artificial *milieu* had changed the properties of the fibres; it is likewise possible that the difference noted was due to the different conditions of recording, against the bath in the one case, against thermally neutral tissue in the other. The nerves may in the former case have had an additional shunting layer of fluid in the bath. Whatever the reasons for the difference it is clear that too much importance should not be attached to the absolute size of the potentials.

As shown by fig. 1 the cooled region became negative upon cooling. For a difference of from 37° to 15° the potential change amounted to about 2 mV. The effect of cooling took place quick-

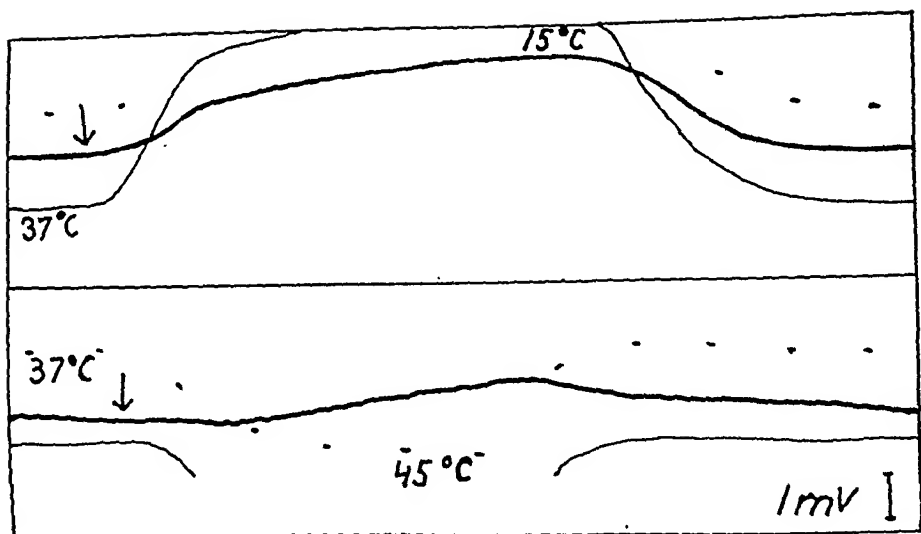


Fig. 1. 7th lumbar motor root, cat. Changes in membrane potential to cooling 37°—15° (upper record) and to warming 37°—45° (lower record). Deflection upwards negativity. Upper temperature line interrupted every 3 sec.

ly and the delay in the appearance of the negativity can probably be wholly explained by the fact that the temperature was not recorded in the nerve but in the affluent branch of the thermode. In these experiments the nerves were only cooled for 15—20 sec. The size of the cold potential, however, is not influenced by the duration of cooling. The nerve may be kept at 15° for half an hour at a constant level of potential, and yet the restitution takes place in the same way as after a much shorter period of cooling. The lower limit in these experiments was at 15° but this value was chosen at random. The depolarization continues at least down to 5° which was the lowest temperature at which observations were made. When the temperature again was increased to its normal equilibrium level at 37° there was often an overshooting relative to its equilibrium value in the restitution of the potential.

When the thermode was warmed from an initial level between 37°—40° the warm region similarly became negative. This potential was of a smaller order. When the nerve was warmed to 45° it amounted to 0.5—1.0 mV. Warming to this temperature has never been extended beyond 10—15 sec. The reason for this is

that when warming had been continued for 15 sec. it was noted that depolarization still was in progress although the nerve must have acquired a constant temperature by this time. This suggests that at 45° a process of deterioration is started and this suggestion was confirmed by the observation that the repolarization for the equilibrium level at 37° was slow and sometimes incomplete.

In the course of an experiment the thermopotentials gradually diminished so that they, after a period of 3 hours, were about 2/3 of their original size.

Dorsal roots were also examined in order to find out whether they differed with respect to their thermopotentials from the ventral roots. The cold potentials were regularly smaller in the dorsal roots while the differences in the heat potentials were less regular. As a rule the latter were larger in the dorsal roots but exceptions were not uncommon.

These observations may be briefly summarized in the statement that the membrane potential of A fibres is equilibrated to the temperature of the body and that both warming and cooling from this level results in a depolarization of the thermally influenced region.

C fibres. In the first experiments on thermopotentials in C fibres the results only differed from those with the roots in that the response to heat was relatively larger than the response to cold. But it soon turned out that the similarity was due to a less satisfactory technique of dissection. As soon as the technique for dissecting the splenic nerves had been perfected it became obvious that in most nerves the thermopotentials in the C fibres differed from those in the roots.

The typical result is illustrated by fig. 2. Upon warming from 37° to 45° a negative heat potential of about 1—2 mV is obtained. On returning to 37° a part of this potential is quickly restituted, another part is restored very slowly. In some cases the fast phase of restitution was missing and the normal level of potential regained very slowly, as in the A fibres.

To cooling from 37° the nerve responded in quite another fashion than the A fibres of the roots. The cold potential began

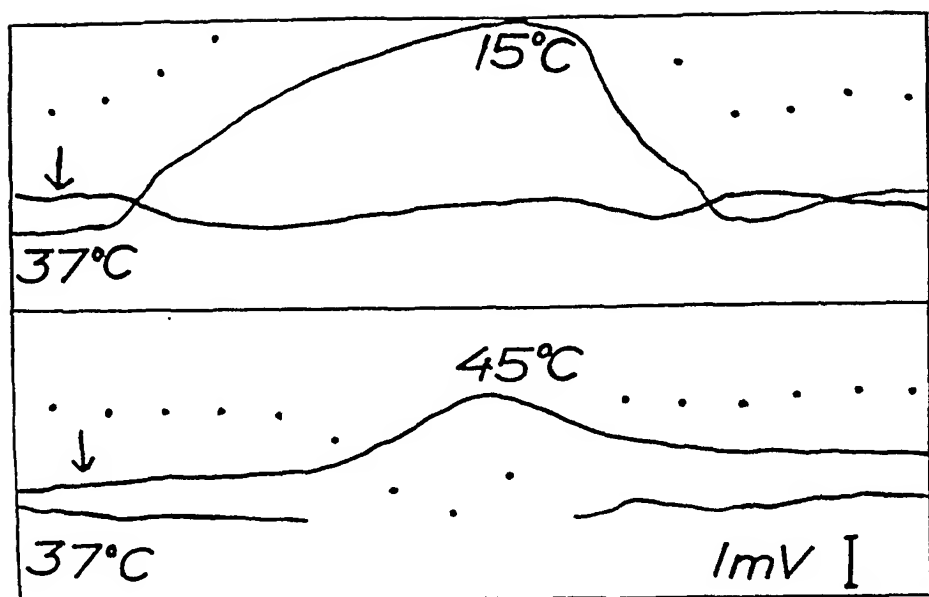


Fig. 2. Splenic nerve, cow. Changes in membrane potential (arrow) to cooling 37°—15° (upper record) and warming 37°—45° (lower record). Deflection upwards negativity. Marked as fig. 1.

with a positivity reaching its highest value at about 25°. When local cooling was continued to 15° this thermopotential diminished and the isoelectric line corresponding to the equilibrium at 37° was attained. But the membrane potential at 15° need not always be precisely at the isoelectric level. It may be somewhat positive or slightly negative. On returning to 37° a positivity is observed which, however, does not become as large as the initial response to cooling from 37°.

Thus the membrane potential of the C fibres has its maximum at a temperature lying considerably below the body equilibrium temperature of 37°.

In order to investigate the thermal equilibrium point — as distinct from the body equilibrium of 37° which for A fibres happened to coincide with the thermal equilibrium point — I used the condenser coupled amplifier with the large time constant of 10 sec. Fig. 3 demonstrates the method employed. The underlying idea is that as long as cooling turns the mem-

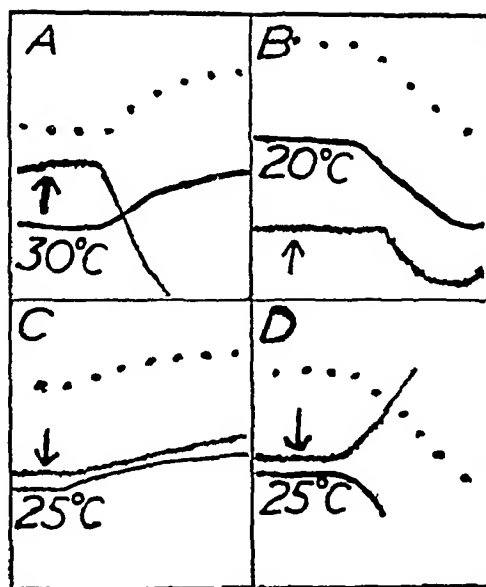


Fig. 3. Splenic nerve, cow. Influence of changes in the temperature on the membrane potential (arrow) studied by means of a condensor coupled amplifier with large time constant and high amplification. Deflection upwards negativity. Cooling from 30° (A) and warming from 20° (B) produce a positive response. To cooling or warming from 25° the response is negative (C resp. D). Upper temperature line interrupted every sec.

brane positive the equilibrium point must be below the temperature from which cooling was started. Similarly, when warming renders the nerve positive, the equilibrium must have been above the temperature from which warming was started. Thus, in the experiment illustrated by fig. 3 when cooling begins at 30° the membrane equilibrium potential must be below 30° because the cooled region has become positive. At 20° local warming has resulted in increased positivity. Therefore the equilibrium point must be somewhere between 20° and 30° . Fig. 3 C and D shows that when starting from 25° both cooling and warming render the region under the thermode negative. The membrane has thus been in thermal equilibrium at 25° .

Experiments with a large number of splenic nerves showed that the thermal equilibrium of the nerve membrane was between 20° and 30° . Mechanical damage shifted this equilibrium point upwards, to higher temperatures. Thus it is possible that

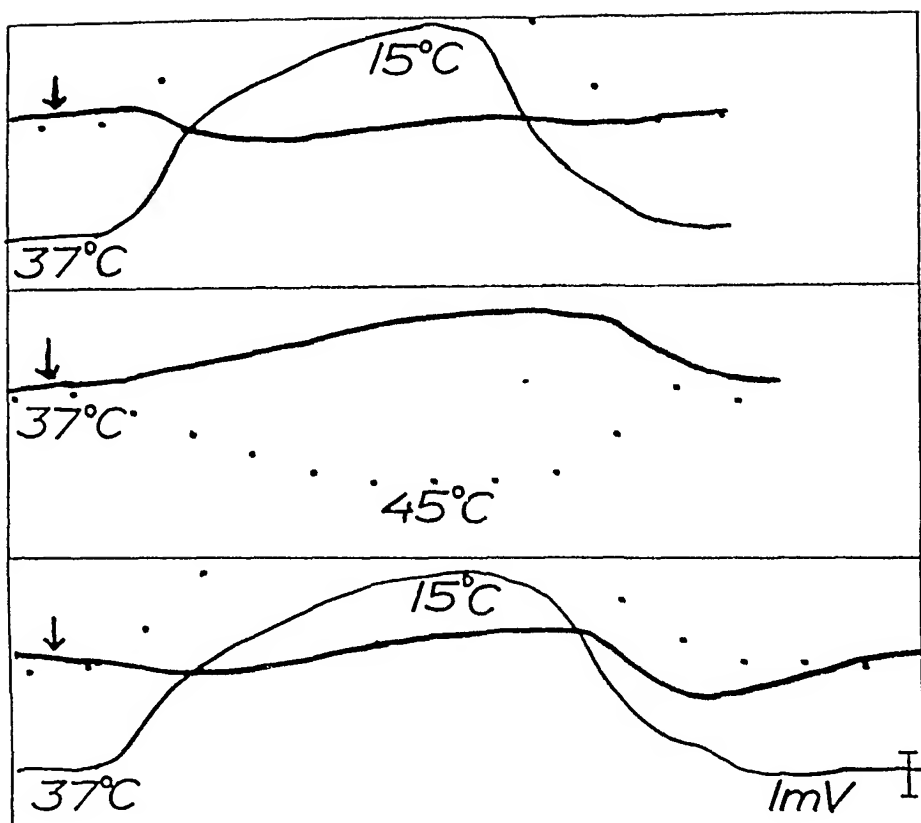


Fig. 4. Splenic nerve, cow. Upper record, normal response of membrane potential to cooling. Middle record, normal response to warming. Lower record taken 1 min. after the middle one shows how the response to cooling after a period warming is altered. Marked as fig. 1.

the lowest values are the most probable ones. This would place the thermal equilibrium point for the splenic nerve at about 20° .

Fig. 4 shows that warming to 45° changes the subsequent cold potential in such a fashion that the initial positivity of the response diminishes, that the nerve becomes relatively more negative at 15° and that upon returning to 37° the positivity increases. A brief heating to 45° is therefore equivalent with damage to the nerve in that both cause a shift upwards of the thermal equilibrium point of the membrane. This indicates that in C fibres heat and cold responses should not be studied in parallel with the thermode at the same region of the nerve. On the other hand,

it should be emphasized that in the splenic nerve neither the thermal equilibrium point nor the thermopotentials themselves change very much in the course of a carefully conducted experiment. After a period of 4—5 hours they are practically the same as in the beginning of the experiment.

Discussion

The results of this section are summarized by the diagram of fig. 5 illustrating the properties of the membrane potential of mammalian A and C fibres as functions of local temperature changes introduced by the thermode technique. The temperature at which the membrane potential has its maximum has been defined as the point of thermal equilibrium. In the diagrams of fig. 5 this point appears as the minimum of the curves for A and C fibres. A glance at these curves shows that, in a general way, results such as those of C. v. EULER (1947) are to be expected. He noticed, it will be remembered, that, starting from body temperature, thin fibres are depolarized by warming to 45°, thick myelinated ones by cooling.

In appraising the significance of these observations it is necessary to consider a few critical objections. In the first place the experiments were carried out on different kinds of mammals. This argument was met by a number of controls devoted to an examination of the thermopotentials in the ventral roots of the cow. These turned out to have the same thermal properties as those of the more conveniently obtainable spinal roots of the cat. In the second place one should consider the circumstance that the C fibres were asphyxiated for a while, whereas the A fibres directly could be put into the nerve bath. Asphyxiation of A fibres was therefore investigated. It was found that after a period of asphyxiation the thermal equilibrium point had shifted downwards to a region of 30—35° but this effect was temporary. It disappeared after some 5 to 10 minutes. Since the C fibres only were asphyxiated for a short while and since their thermal equilibrium was permanently low it is impossible to ascribe the differences between A and C fibres to this particular experi-

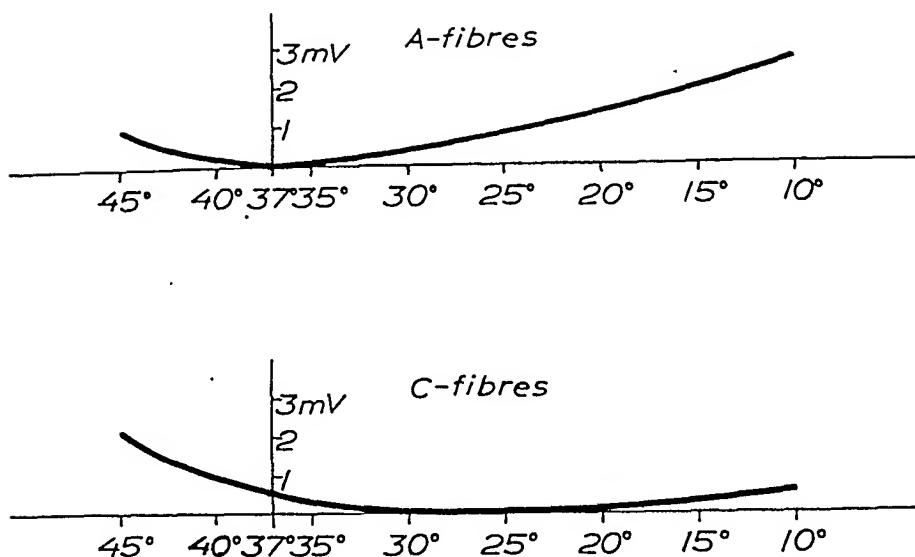


Fig. 5. The membrane potential of A and C fibres as functions of temperature. The temperature ranges for minimum ordinates represent the equilibrium point (=maximum of membrane potential).

mental factor. Thus, there is every reason to believe the differences described to be real.

It is accordingly concluded that the motor A fibres are thermally equilibrated to body temperature — as indeed a priori would have seemed natural for all nerves — and the C fibres to a considerably lower temperature.

Chapter 2. Differences in thermal sensitivity between the action potentials of mammalian A and C fibres

In the former chapter it was shown that the membrane potentials of mammalian A and C fibres possess their optima at different temperatures. The depolarizations themselves set up by moderate temperature changes, are small and need not necessarily do more than slightly modify the functional activity of the nerve. Nevertheless rather remarkable alterations of nerve functions are caused by temperature changes which but little can have changed the membrane potential (VERZAR 1911, BREMER and TITECA 1934, GASSER 1931, LORENTE DE NÓ 1947). The differ-

ences found in the thermal equilibria of A and C fibres, though interesting in themselves, need not therefore represent significant functional differences between these two fibre types. For this reason it is important to examine some other aspects of nerve activity such as spike height and afterpotential in order to establish whatever temperature correlations can be established by these means. In this chapter a beginning is made with the spike.

The most interesting results have been obtained by cooling the nerve. Warming has only been investigated up to 45° as higher temperatures tend to cause prolonged and profound alterations of the thermosensitivity of the nerves. The heat block, so readily accessible to analysis in cold-blooded animals, has therefore not been included in the present work. The nerve is irreversibly damaged at temperatures which are high enough to block the impulse.

A fibres. In the motor alpha fibres the maximum spike height was obtained around 30° — 35° . Both cooling and heating from this range reduced it. As shown by fig. 6 the changes due to cooling from 37° are, to begin with, small. But further decrease in temperature brings on a rapid diminution of the spike which entirely disappears at temperatures between 15° — 7° , variable from case to case. The effect on the shape of the spike will be discussed in Part II.

It has been shown that in frog nerves the blocking temperature for cold increases when the nerves have been mechanically damaged even though this damage has been of a very modest order (Boyd and Ets, 1933). This, in my experience, is true also of mammalian nerves and it is possible that the variations in sensitivity to cooling can be referred to the degree of damage to which the nerves have been exposed during the dissection. It was necessary to dissect free a part of the extradural portion of the root in order to obtain nerves of sufficient length. Despite every care exercised in this operation it is difficult to perform it with the full assurance that slight mechanical damage has been completely avoided. For this reason it is likely that the lowest values found in these experiments are the most probable ones. There is a definite connexion between blocking temperature and the

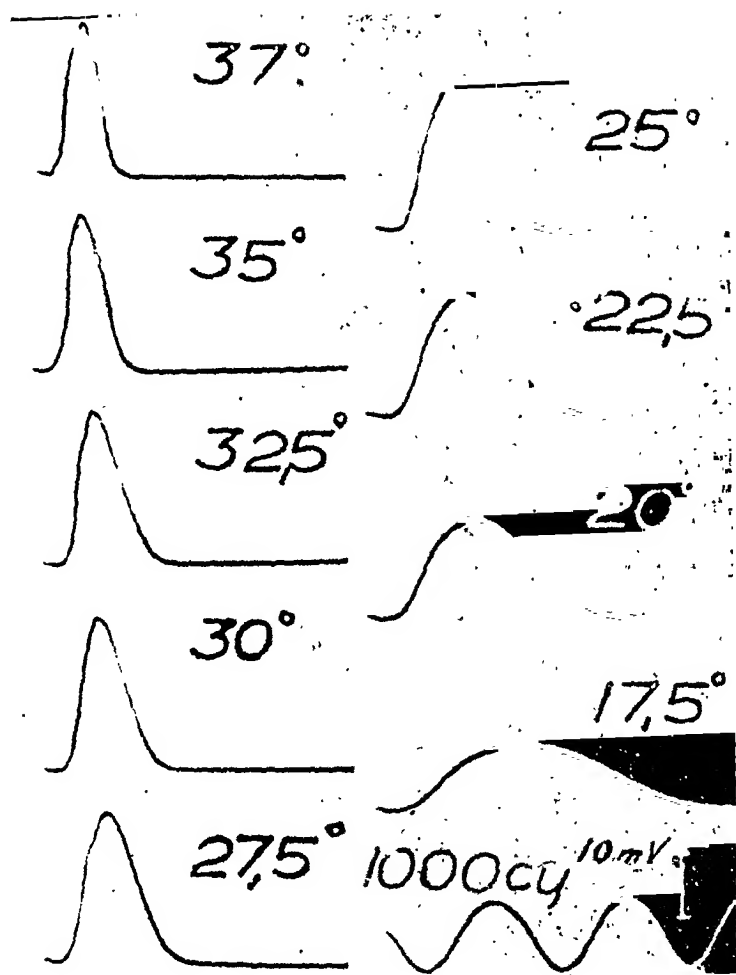


Fig. 6. 7th lumbar motor root, cat. Supramaximal stimulation of alpha fibres. Spike recorded as different temperatures. Interpolar distance of recording electrodes 15 mm.

optimum temperature. When one is raised or lowered the other one follows suit.

The negative afterpotential has also been included in this analysis of temperature effects.

LORENTE DE NÓ (1947) found that his L-fraction is small in freshly excised mammalian nerve and that it, despite the presence of CO_2 (which favours the L-fraction), after a couple of hours had still further decreased. Since the negative afterpoten-

tial is a function of the L-fraction one would expect it to behave in a similar fashion and this, in fact, is what I found. The negative afterpotential in the roots disappeared after some hours. It was then replaced by a positive deflexion. For this reason the temperature sensitivity of the negative afterpotentials was examined in the course of the first hours of the experiment.

Fig. 7 illustrates a typical experiment. The negative afterpotential has an optimum in the temperature range 37° — 40° . The left part of the record of fig. 7 shows the response to cooling from 37° . The negative afterpotential diminished rapidly and disappeared already at 27.5° . In some cases it was slightly more resistant to cold but I have never seen it withstand 20° . When the root was warmed after a period of cooling it exceeded its normal value at 37° (uppermost record, on the right, one minute after this temperature had been established). The rest of the figure shows that the negative afterpotential again diminished when the nerve was warmed from 40° . Thus the maximum was in the temperature range of 37° to 40° .

In addition to the standard preparations of motor roots I have also employed sensory roots as well as the phrenic and peroneal nerves. The A spikes of the dorsal roots proved less sensitive to cooling than those of the more homogeneous motor roots (pure alpha). The maximum spike height was at 20° — 30° and the spike disappeared between 5° — 10° . The lowest blocking temperature noted was 3° . In the peripheral nerves the spike height was maximal at 25° — 15° and the spike disappeared at 3° — 0° . In the peripheral nerves the negative afterpotential of the alpha fibres was maximal at 30° — 37° .

The differences in thermosensitivity just described cannot at the present moment be fully explained. There may be real differences between mammalian motor and sensory fibres and there may be a gradient in thermosensitivity along the nerves. These questions will have to be taken up in a separate paper. In this work the motor alpha fibres will serve as the prototype for A fibres.

C fibres. The properties of the C fibres are less constant than those of the A fibres and so provide greater experimental dif-

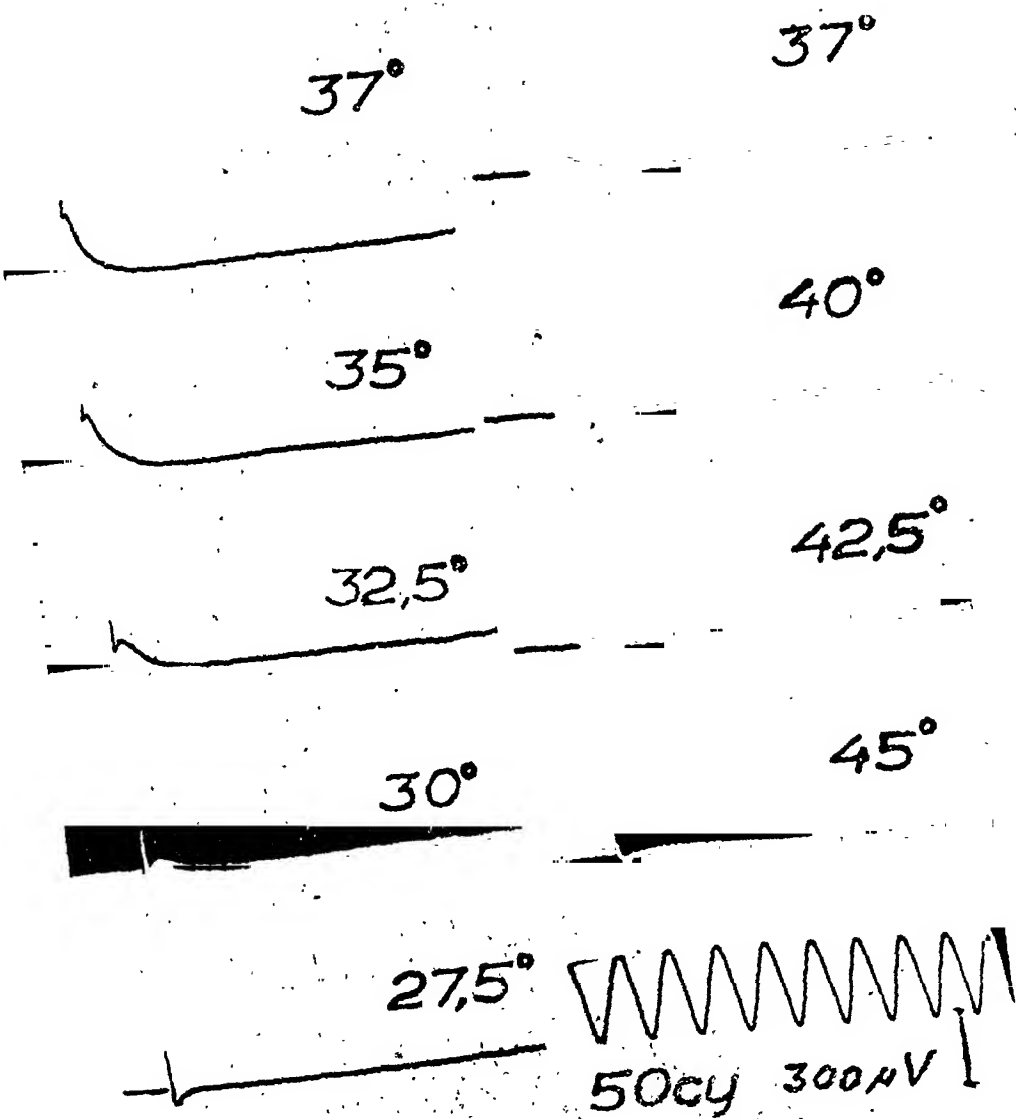


Fig. 7. 7th lumbar motor root, cat. Afterpotentials of alpha fibres; supra-maximal stimulation. Records at different temperatures. Interpolar distance of recording electrodes 15 mm.

ficulties. Attention to humidity is of the utmost importance. The C fibres are extremely sensitive to drying, particularly after removal of the perineural sheaths.

In investigating the action potentials at low temperatures certain precautions have to be observed. Below 15° a further

decrease of temperature lengthens the absolute and relative refractory periods. The latter may at the very low temperatures actually be as long as 10 sec. Therefore it has been necessary to stimulate cooled C fibres with shocks not less than 30 sec. apart. But already above 15° care should be exercised in not stimulating too frequently. If this is done one observes an increased negative afterpotential and a corresponding increase in spike height. This is probably due to a lengthened positive afterpotential. In order to prevent an accumulation of this change, C fibres, in the range of 37° — 15° , have been stimulated by shocks not less than 10 sec. apart.

With supramaximal stimulation of C fibres a considerable increase of spike height ensues upon cooling (fig. 8). The effect can be shown to be restricted to the cooled region. The spike height does not increase just outside the thermode. The temperature for maximum spike height varies somewhat in different nerves but generally lies between 5° — 10° . The increase may then amount to 300—400 %. Upon further cooling a diminution takes place. In most nerves the spike does not disappear until a temperature below -5° has been reached. In some nerves, however, the spike disappeared already between 15° — 20° . There is a similar correlation between blocking and optimum temperatures here as for A fibres. The higher the one, the higher the other. We have every reason to ascribe the shift upwards of these two temperature functions to a similar cause, i.e. to some damage making the nerves less resistant to cold. Accordingly the lowest temperatures noted should be considered the most physiological ones, the more so as the shift upwards of the temperature functions characterized a relatively small number of the preparations that were investigated from this point of view.

The negative afterpotentials in the splenic nerve also have thermal properties differing from those of the A fibres. In most nerves the afterpotentials had their maximum between 25° — 30° but some exceptions were noted. Fig. 9 illustrates an experiment in which the negative afterpotential of the splenic nerve was maximal at about 25° . Cooling causes spectacular changes in the amplitude of the negative afterpotentials. There is a definite increase already in response to a temperature drop of a few degrees.

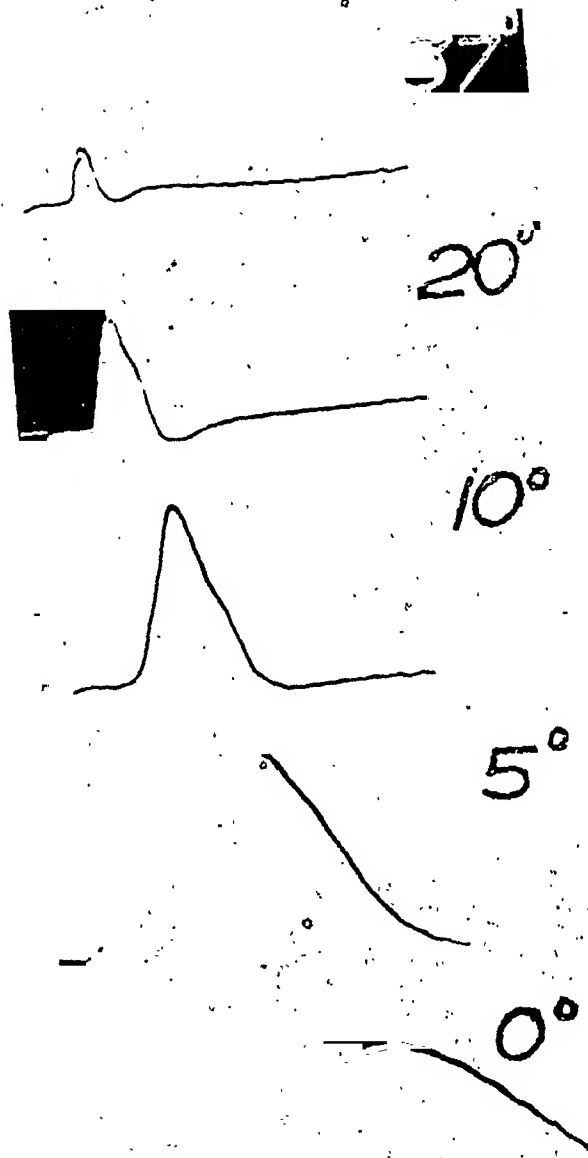


Fig. 8. Splenic nerve, cow. Supramaximal stimulation of C fibres, spike recorded at different temperatures. Interpolar distance of recording electrodes 15 mm.

50cy 1mV I

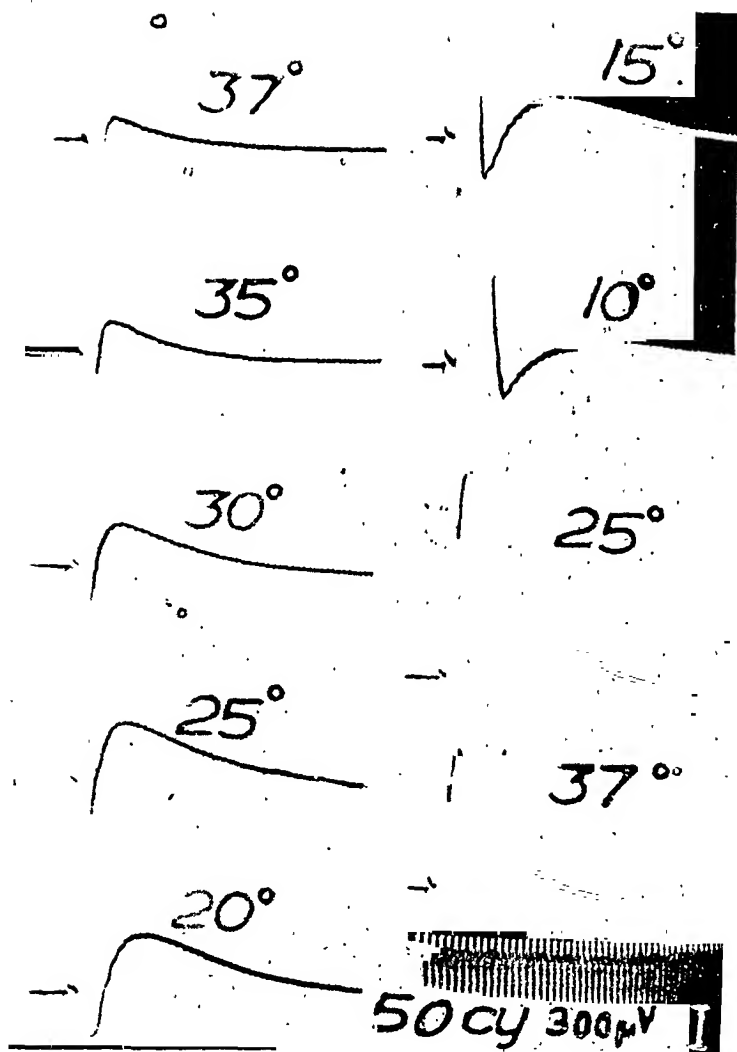


Fig. 9. Splenic nerve, cow. Supramaximal stimulation of C fibres. Afterpotentials recorded at different temperatures. The two lowermost records to the right, labelled 25° and 37°, taken upon returning from a period of cooling to 7.5°. Interpolal distance of recording electrodes 18 mm.

At 25° the increase in the negative afterpotential has reached its maximum value; it then diminishes but does not disappear completely until a temperature of 7.5° has been reached. Usually the negative afterpotential did not withstand cooling to such a low temperature but disappeared between 15°—10°. The figure also shows that there is a considerable increase of the duration

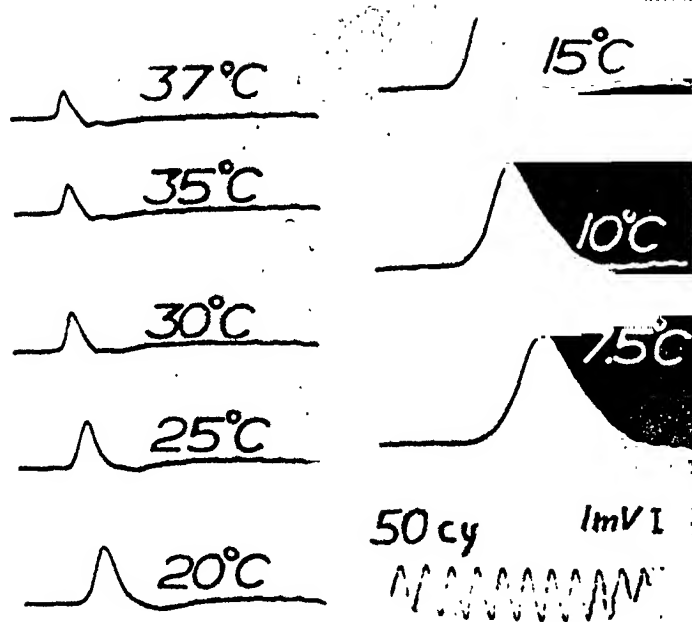


Fig. 10. Splenic nerve, cow. Same nerve as in fig. 9. Records taken one hour later. Supramaximal stimulation of C fibres. Interpolat distance of recording electrodes 18 mm.

of the negative afterpotential. Fig. 9 further illustrates that re-warming (in this case from 7.5°) also causes a large increase of the amplitude of the negative afterpotential. This increase which is found in most nerves persists a few minutes. The same phenomenon was observed with the A fibres but it is far more conspicuous in the C fibres. Fig. 10 illustrates the spike as a function of temperature in the same nerve as that used for the experiment illustrated by fig. 9.

Even though every care has been exercised in order to maintain proper humidity one sometimes finds the maximum of the negative afterpotential at a very much higher temperature than normally and occasionally it is also impossible to provoke a negative afterpotential, even by cooling. In such cases it is interesting to observe that the effects of cooling on the spike and on the afterpotential may run a different course.

In order to discover whether the differences between mammalian A and C fibres, described in this section, also could be

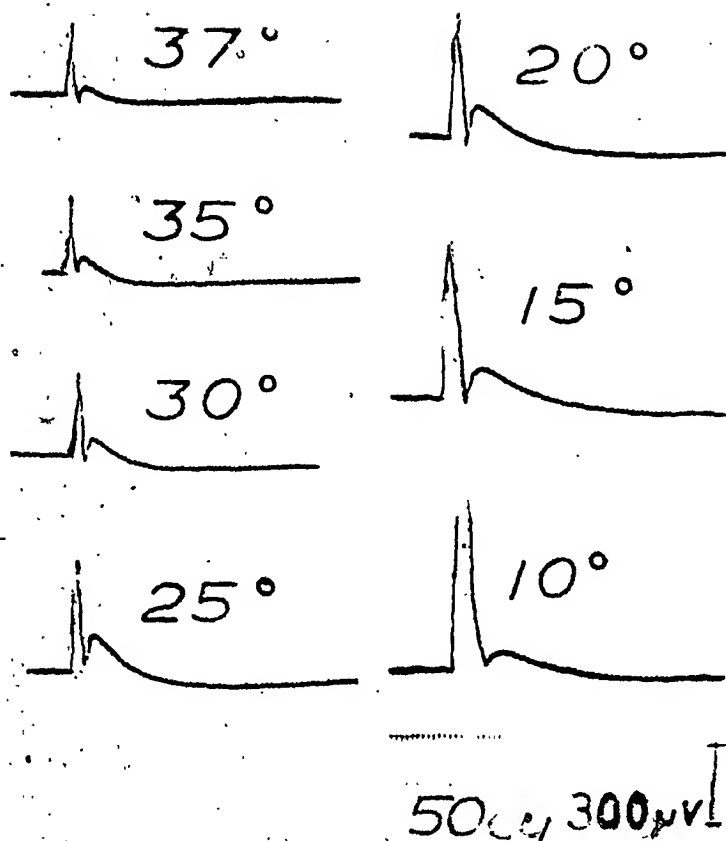


Fig. 11. Hypogastric nerve, cat. Supramaximal stimulation of C fibres. Action potential recorded at different temperatures. Shock artefact and B spike seen as discontinuity and irregularity of the ascent of the C spike. Interpolated distance of recording electrodes 15 mm.

reproduced in the same species, experiments were carried out with the excised hypogastric and accelerans nerves of the cat. They can be quickly excised and treated in the same way as the roots and that is of course satisfactory from a physiological point of view, but on the other hand the thin nerves are easily damaged during dissection and, as we have seen, injury of the slightest kind is sufficient to shift the temperature function upwards. However, in most of those nerves the response to cooling agreed with the effect just described for the splenic nerve of the cow. Fig. 11 illustrates a hypogastric nerve with the maximum of the

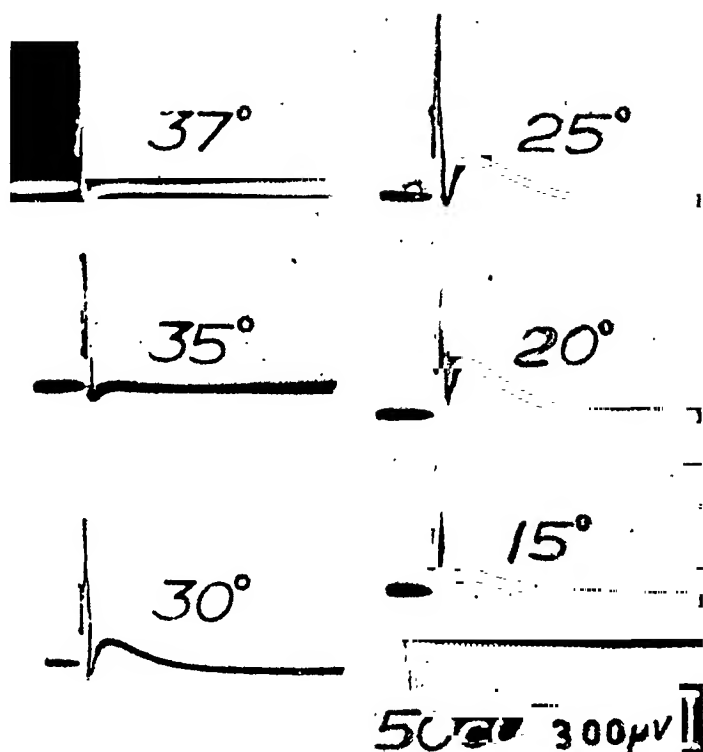


Fig. 12. Hypogastric nerve, cat. Supramaximal stimulation of C fibres. Action potential recorded at different temperatures. Interpolar distance of recording electrodes 15 mm.

negative afterpotential at 25° and with a 250 % increase of spike height at 10°.

It was mentioned above that the response to cooling of the spike could be normal despite the fact that the negative afterpotential was absent or was equilibrated to a higher temperature than normal. Fig. 12 demonstrates the opposite relationship. The response of the negative afterpotential to cooling is quite normal but spike height has its maximum at 25° instead of around 5°—10°. Spike height and negative afterpotential are thus different functions of the temperature, and these functions may be independent of one another.

Simultaneous measurements of temperature potentials and the variations of the action potentials in response to cooling of the splenic nerve were performed in a number of cases. When the

nerve was cooled from 37° to the temperature at which the negative afterpotential reaches its maximum it was observed that, if the negative afterpotential in the course of such an experiment increased but modestly, then too the positivity that initiated the membrane response to cooling was small. It was also found that the membrane potential had its maximum in the same range of temperature as the negative afterpotential. Thus when a splenic nerve was chosen with the maximum afterpotential shifted upwards the membrane equilibrium was also shifted to the same range of temperature.

Discussion

The experiments have shown that A and C fibres differ with respect to the temperature sensitivity of membrane potential, spike and negative afterpotential. In motor alpha fibres the membrane potential has its maximum around body temperature; similarly the negative afterpotential, but spike height is maximal at 30° — 35° . In the C fibres the membrane potential and the negative afterpotential likewise have maxima in the same region, in this case, however, around 25° , and spike height is maximal at a temperature as low as 5° .

The criticism that could be raised against the experiments on membrane potentials, namely that the A and C fibres were from different species (cow, cat), is irrelevant with respect to the spikes and afterpotentials because the C fibres of the cat were shown to behave like those of the cow.

Another objection concerns the fact that A fibres from the roots have been compared with C fibres in peripheral nerve. It was shown already by DU BOIS-REYMOND (1849) and recently confirmed by REXED (1947) that the demarcation potential diminishes from a maximum value in the roots downwards along the nerve. It is impossible to exclude a gradient in thermosensitivity. Actually the experiments showed that the action potential in the root had a different temperature sensitivity than further down the nerve. But this effect may be due to sensory fibres, to a real gradient of thermosensitivity or to both factors acting together. Further experiments should therefore be devoted to investigating

potential than the L-component since the negative afterpotentials at 37° are small or absent.

It has been shown that in a normal splenic nerve cooling from 25° causes some depolarization of the membrane but a considerable increase of spike height. This great increase in spike height is possibly a real increase of the internal action potential. These results do not fit the old theory according to which the spike potential is a passive and relatively complete depolarization of the membrane potential. There is also direct evidence against it. Thus HODGKIN and HUXLEY (1939; 1945) and COLE and CURTIS (1941) have shown that in evertibrate nerve the spike potential exceeds the membrane potential, and LOR-ENTE DE NÓ (1947), with frog nerve, has found that the impulse cannot be regarded merely as a temporary disappearance of the resting electromotive forces but represents the creation of new electromotive forces tending to establish a membrane potential of a sign opposite to that of the resting potential. The large increase of the spike in the cooled C fibres is therefore probably due to altered conditions for these electromotive forces, to be accounted for by the low temperature.

PART II

The thermal sensitivity of A and C fibres in relation to the concentration of potassium ions

Introduction

It is evidently an attractive programme of research to investigate the effect of various substances on the thermal sensitivity of A and C fibres in order to obtain further information about the mechanisms concerned. Preliminary experiments had shown that potassium ions influenced the thermal sensitivity of the membrane potential and for this reason a beginning was made with experiments varying the concentration of potassium in the medium of the nerve.

Historical section

It has long been supposed that potassium ions are of great importance for the nerve fibre. The greater concentration of potassium inside than outside the nerve fibre led BERNSTEIN (1902, 1912) to his well-known membrane theory according to which the membrane potential was explained by this concentration difference on the supposition that the membrane itself was impermeable for sodium ions and anions. It was noted by MAC DONALD (1900), HÖBER and STROHE (1929), and BISHOP (1932) that addition of potassium to the external medium caused depolarization but the theory also required that the depolarization potential should be a linear function of the logarithm of the potassium concentration. COWAN (1934), STEINBACH (1940) and COLE and CURTIS (1942) found this to be the case for a limited range and with vertebrate nerves but their results were not confirmed by GUTTMAN (1940). LORENTE DE NÓ (1947),

working with frog nerve, did not find a linear relationship between the logarithm of potassium concentration and the amount of depolarization.

LORENTE DE NÓ (1947) also produced evidence for the inadequacy of the classical theory and himself arrived at the conclusion that the potential difference across the membrane is maintained directly by the oxidative metabolism. FENN, COBB, HEGNAUER and MARSH (1934) have shown that, when a frog nerve is kept in a solution containing 0.005 M potassium, the internal potassium concentration of the nerve remains constant but that every increase or decrease of this concentration makes the nerve respectively gain or lose potassium ions.

LORENTE DE NÓ (1947) has summarized his own results in the following statement: "The external concentration of K^+ ions plays a rôle in the maintenance of the resting membrane potential only in so far as it is necessary for the maintenance of the internal potassium concentration, or otherwise stated, in so far as the changes in the external result in changes in the internal potassium concentration. The rôle that is played by the latter is more direct but it is subordinate to the rôle of oxidative metabolism, because although an increase of the internal potassium concentration results in a depolarization of nerve, the membrane potential may be increased even beyond the normal level by the exalted activity of the oxidative mechanisms that follows after a period of anoxia. Thus, it can be stated that oxidative metabolism may result in the establishment of a membrane potential of the same magnitude as that of untreated nerves in the presence of a lowered internal concentration of potassium as well as in the presence of a raised concentration."

The rôle played by the high internal concentration of potassium is unknown but probably it serves to maintain the internal milieu required by the metabolic processes. CHANG, SHAFFER and GERARD (1935) have shown that an excess of potassium ions in the external medium lowers the resting oxygen uptake. LORENTE DE NÓ (1947) has found that, at least in a certain phase, excess potassium in the external medium diminishes the rate of the anoxic depolarization of the nerve. This is of importance because he has also found that agents which delay the anoxic depolari-

zation retard some of the metabolic processes engaged in maintaining the membrane potential. In some experiments Lorente de Nó investigated the effect of potassium-free Ringer. In respiring nerve the membrane potential was not significantly altered but in anoxic nerve he observed a temporary delay of the depolarization, and the oxidative repolarization was accelerated, compared with the rate of repolarization in normal Ringer. These results suggest that by a diminution of the potassium concentration different conditions had been established for the metabolic processes.

The literature concerning the significance of the potassium/calcium ratio for nerve functions is extensive. Only some observations on the action potential will be mentioned below.

H. T. GRAHAM (1933) studied the effect of cations on frog nerve. She found that an augmentation of the potassium ion concentration in normal Ringer of from 3 to 10 times diminished both the spike height and the negative afterpotential and that the latter underwent a relatively greater decrease. Augmentation of the Ca-concentration by 9 to 13 times augmented the amplitude of the negative afterpotential and its duration whereas the spike height remained practically unchanged. The question concerning the K/Ca balance was taken up again by GRAHAM and BLAIR (1947) in experiments on frog nerves. Spike height, afterpotential and several other functions were investigated. They found that, when the increase of K was above 2.5 times the standard concentration, the nerve functions changed in a manner characteristic for K-ions. If, however, the nerve was placed in a solution with augmented Ca- or diminished K-concentration the functions altered in an opposite direction. They explained their results by assuming that the functions investigated to a greater or lesser degree were controlled by the K/Ca ratio and that the Ca-ion lost its capacity of counteracting potassium when the K-concentration increased above a certain concentration thereby causing specific effects on the nerve.

LEHMANN (1937) studied variations in the K/Ca-balance on the phrenic and peroneal nerves of the cat. He found the effects of a changed K/Ca ratio to appear very slowly. An 8-fold increase of the concentration of either ion, acting on the nerve for 1

hour, caused but small changes in the action potential. A solution lacking Ca-ions, acting for a couple of hours, made the nerve discharge spontaneously and the afterpotentials became rhythmic. An increase of the potassium concentration caused similar effects though less marked. If the nerve for 8 hours was subjected to the influence of KCl-free Krebs solution changes similar to those described by H. T. GRAHAM (1933) for frog nerves treated with excess Ca were obtained, *i. e.* an augmentation of the negative afterpotential and its duration.

LORENTE DE NÓ (1947) has also studied the effect of an increase of the concentration of potassium ions on the action potential of frog nerve. He found that the negative afterpotential, the L-fraction, soon disappeared. Of great interest is that the effect of potassium could be counteracted by CO₂ so effectively that a conduction block set up by excess potassium was abolished by treatment of the nerve with this gas. Again, from the point of view of my own results, it is of especial interest that an increase of the K-concentration first affected the A fibres, then the B fibres and ultimately the C fibres although this differentiation was noted with the groups rather than with individual fibres. Thus, for instance, there were A fibres which were more resistant to the influence of potassium than the least resistant C fibres.

Very few observations on the effect of potassium upon the thermal sensitivity of nerve seem to have been published. ETS and BOYD (1933) noted that the temperature necessary for a cold block in frog nerves was raised by an increase of the potassium ion concentration. And in the same paper it is stated that Ca does not counteract this particular effect of potassium.

Methods

For the recording of the membrane potential and the action potential the same methods as in the first section have been used. The solutions with changed concentration of potassium have been applied locally on the nerve within the thermode. For changes in the potassium concentration up to 100 % an isotonic KCl-solution has been added to the normal Krebs

solution. Changes above 100 % have been obtained by replacing part of the NaCl with KCl. Solutions lacking potassium have been obtained by replacing KCl with NaCl and wholly removing KH_2PO_4 . Controls have shown that the absence of phosphate ions does not influence the thermal sensitivity.

Results

Chapter 1. Potassium and the thermal sensitivity of the membrane potential of A and C fibres

In these experiments the effect of potassium was investigated on the roots, or, if peripheral nerves were used, on nerves from which the perineural sheath had been removed. BISHOP (1932) found with frogs that potassium ions affect the roots much sooner than the nerves. I have confirmed this observation on cats. Thus, for instance, in one experiment a 100 % increase of the K-concentration in the solution applied onto a root caused a marked increase of excitability within 10 sec. whereas application of the same solution on a peripheral nerve with intact sheath only led to a small effect within 15 minutes after which it increased very slowly. In de-sheathed peripheral nerve the effect was faster but still not as fast as in the root.

Even though these experiments did not suffice to demonstrate that the sheaths were an obstacle to the penetration of K-ions into the nerve fibres they nevertheless served to indicate that this might be the case. For this reason I preferred to use roots or de-sheathed nerves when trying to elucidate the effect of relatively modest changes in the potassium ion concentration of the external medium.

A fibres. In the beginning potassium concentrations were chosen which had been shown by other authors to influence frog nerve.

By increasing the potassium concentration of a Krebs solution 4 times above the normal value the temperature sensitivity of

the membrane potential was changed so that cooling from 37° to 15° , instead of causing its normal depolarization of about 2mV, only caused a change of 0.5—1.0 mV. To warming from 37° to 45° the root did not respond at all by the characteristic depolarization but the nerve became slightly positive by about 0.1 mV.

Doubling the normal potassium concentration gives a different picture, as illustrated by fig. 13. Cooling from 37° to 15° produced much the same depolarization as before but warming to 45° caused a positive response of up to 1 mV instead of the characteristic depolarization seen in normal roots. The maximum of the positive response to warming may be reached already below 45° and further warming may lead to depolarization. On returning to 37° one finds the membrane potential not only return to its original level but actually pass below it so that the nerve becomes depolarized relative to its status at 37° . This is illustrated by the experiment of fig. 13.

What has happened in this case is that the membrane potential under the influence of the increased potassium concentration of the external medium has become equilibrated to a higher temperature. An increase of only 50 % was found to place the equilibrium of the membrane around 40° — 42° . Further increase of the potassium concentration shifted the equilibrium temperature of the membrane to the region around 41° — 45° .

If the nerve be washed with a Krebs solution lacking potassium ions interesting changes in the sensitivity of the membrane to thermal variations occur (fig. 14). The previous depolarization to cooling from 37° becomes replaced by repolarization, a positive response later, on further cooling, supplanted by a negativity so that ultimately at 15° , the membrane potential has about the same value as at 37° . In other words, the A fibres treated by a potassium-free solution assume the properties of C fibres with respect to the membrane potential. The response to warming from 37° — 45° also changes; one obtains a large depolarisation of from 1.0 to 2.0 mV.

Thus, in the nerves treated with potassium-free solution, the membrane potential becomes equilibrated to a lower temperature, generally a temperature between 25° — 30° . Fig. 15 gives a

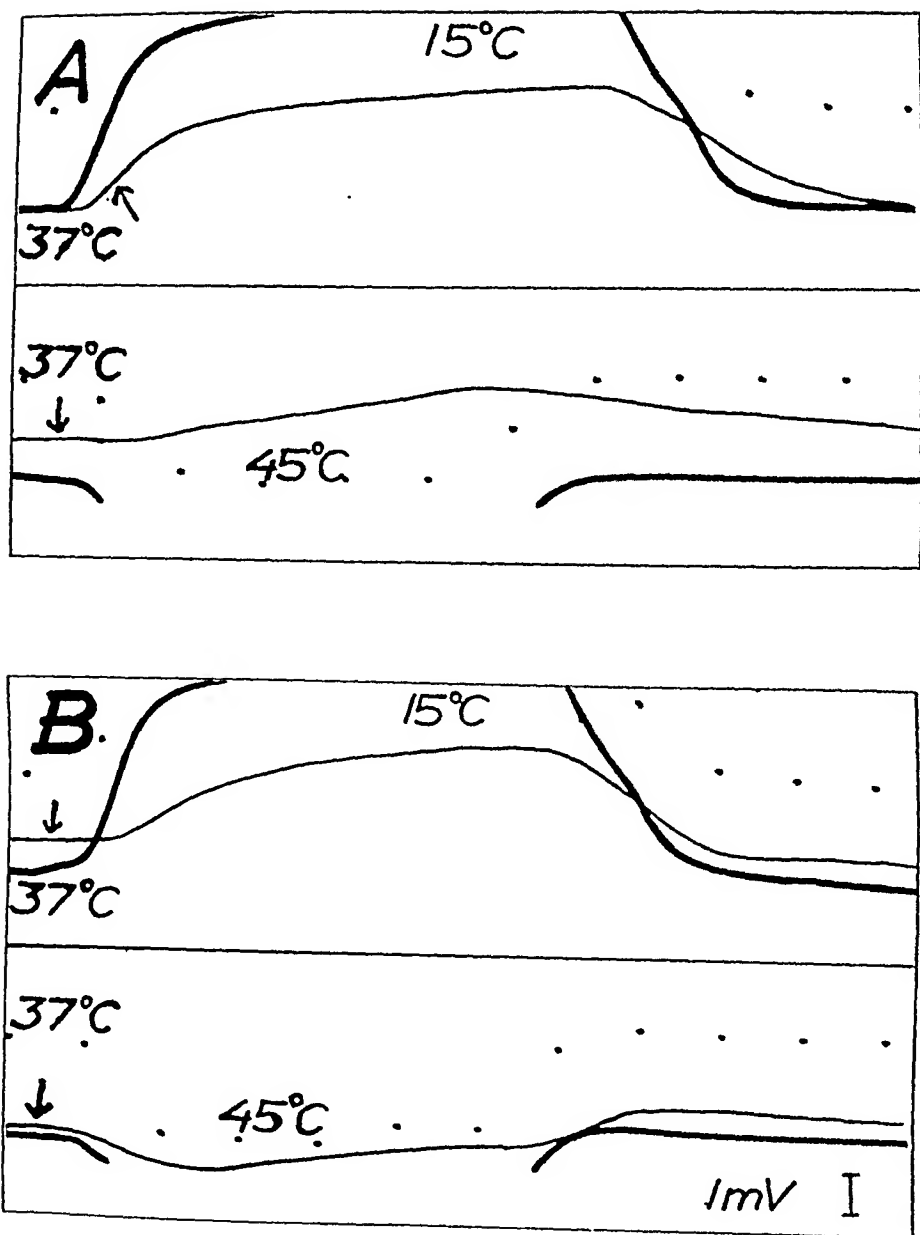


Fig. 13. 7th lumbar motor root, cat. Response of membrane potential (arrow) to cooling 37°—15° and to heating 37°—45°. Deflection upwards negativity. *A*: root which had been kept in normal Krebs. *B*: the same root treated 10 minutes with Krebs solution in which the concentration of potassium had been doubled. Upper temperature line interrupted every 3 sec.

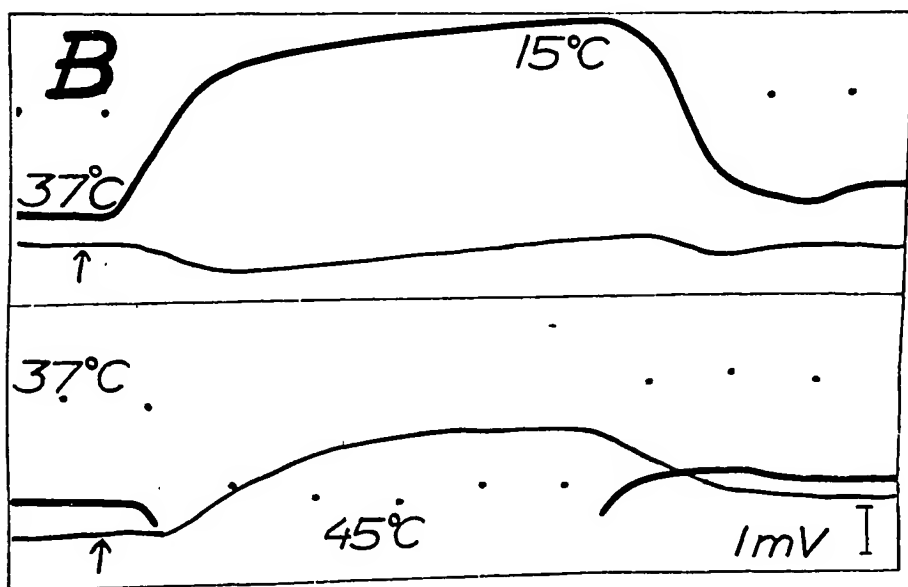
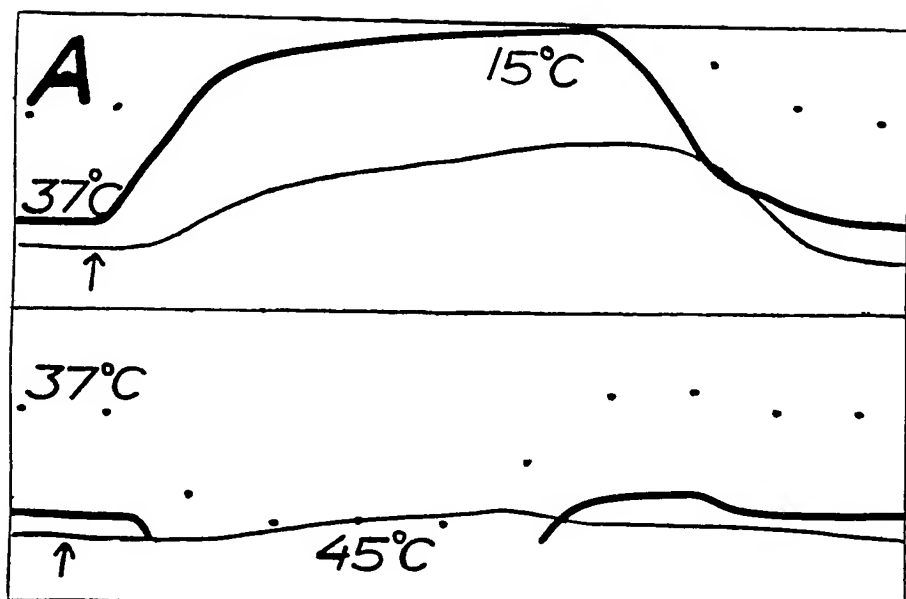


Fig. 14. 7th lumbar motor root, cat. Response of membrane potential (arrow) to cooling 37°—15° and to warming 37°—45°. Deflection upwards negativity. *A*: root which had been kept in normal Krebs. *B*: the same root treated 10 minutes in Krebs solution lacking potassium. Marked as fig. 13.

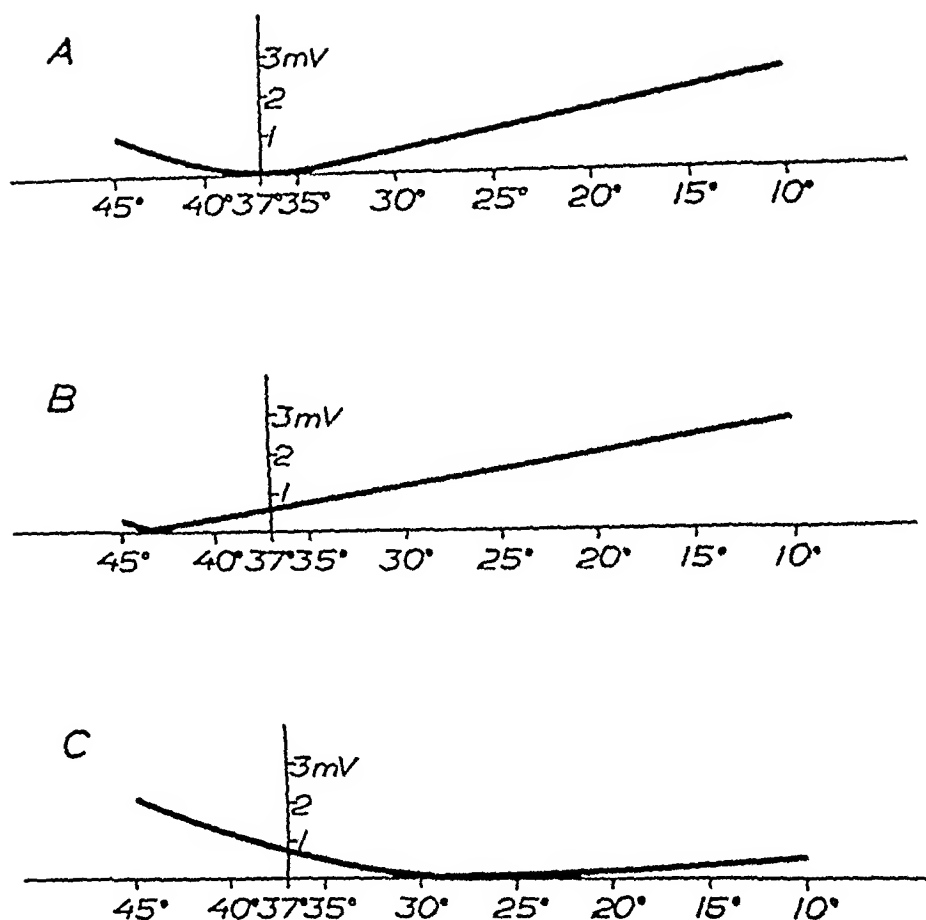


Fig. 15. Membrane potential in motor root as function of temperature. A: normal root. B: root treated in Krebs solution containing twice the normal amount of potassium. C: root treated with potassium free Krebs. The temperature range for minimum ordinates represent the equilibrium point (=maximum of membrane potential).

schematic picture of the changes in the temperature sensitivity of the membrane caused in A fibres by variations in the potassium concentration of the external medium.

C fibres. Also in the C fibres (the splenic nerve of cow) radical changes in the sensitivity of the membrane occur when the concentration of potassium ions in the external medium is varied. Fig. 16 illustrates that an increase of the potassium concentration causes a change in the direction of the type of reaction character-

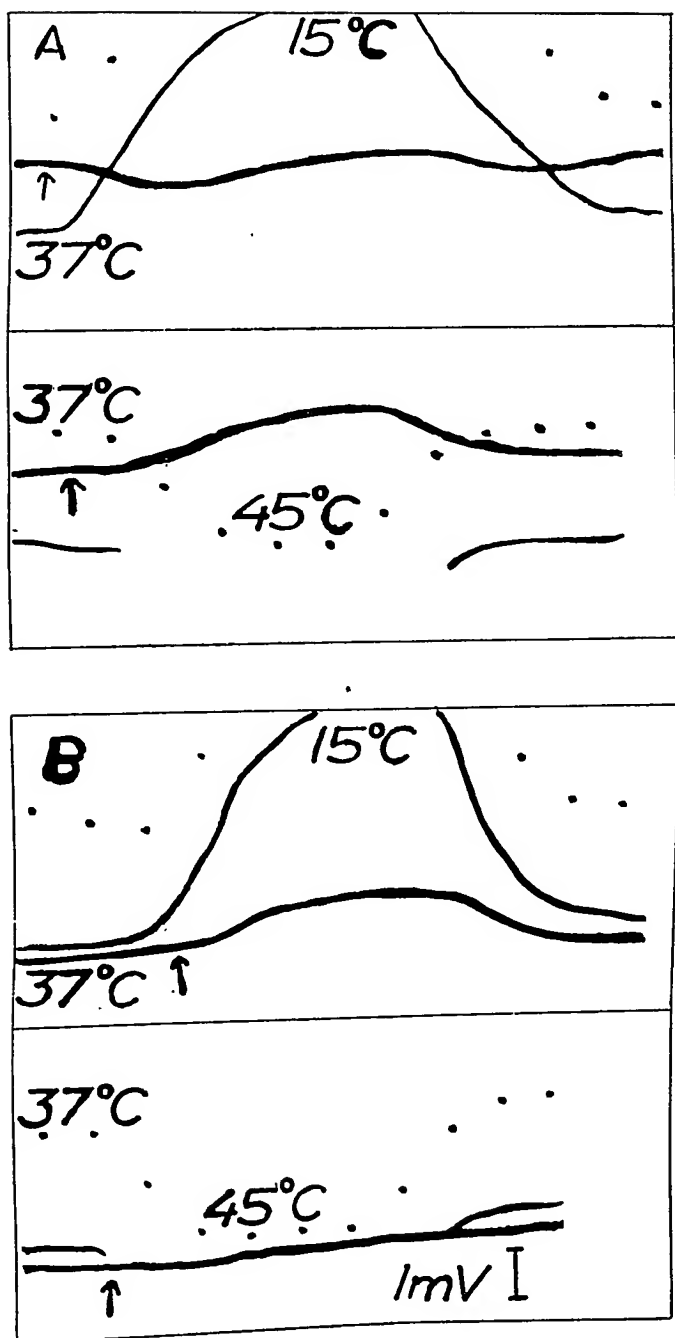


Fig. 16. Splenic nerve, cow. Response of membrane potential (arrow) to cooling 37°—15° and warming 37°—45°. Deflection upwards negativity. *A*: nerve which had been kept in normal Krebs. *B*: the same nerve treated during 30 minutes with Krebs solution containing 2.5 times the normal concentration of potassium. Marked as fig. 13.

ising A fibres. The normal positivity to cooling from 37° has been replaced by a depolarization. In the experiment illustrated in fig. 16 this depolarization hardly amounts to 1.0 mV at 15° but it may be increased by increasing the potassium concentration. The large depolarization set up by warming to 45° has almost disappeared. It is always greatly diminished.

Thus an increase of the concentration of potassium influences A and C fibres in the same way. In both cases there is a shift of the thermal equilibrium temperature upwards. The increase necessary for shifting the thermal equilibrium of C fibres from the normal range around 25° to that of the A fibres around 37° varies from nerve to nerve but is of the order of 100–200 %. In many cases one observes that an increase of 100 % shifts the equilibrium point to 37° but that the depolarization to cold then is relatively small and tends to increase with a further increase of the potassium concentration.

Treatment of the splenic nerve with a Krebs solution lacking potassium ions changes the membrane response to temperature in the manner illustrated by fig. 17. Cooling from 37° produces a larger positivity than normally and, at 15° , the membrane is regularly positive relative to its value at 37° . The membrane potential has its largest value at a temperature between 15° and 20° .

A diagram (fig. 18) corresponding to the one for A fibres (fig. 15), illustrates for C fibres the shift in the thermal equilibrium point of the membrane potential as a function of the potassium concentration of the solution.

It deserves to be emphasized that both for A and C fibres all the effects described are fully reversible and that normal conditions are established as rapidly as effects of changed potassium concentration when afterwards the nerve again is treated with normal Krebs solution.

In these experiments I have not systematically measured the times necessary for obtaining full effects of the changed *milieu* but I have observed that with roots the altered response, as a rule, was fully developed after 5–10 min. independently of whether the amount of potassium ions was augmented or diminished. With the splenic nerve somewhat longer times were necessary.

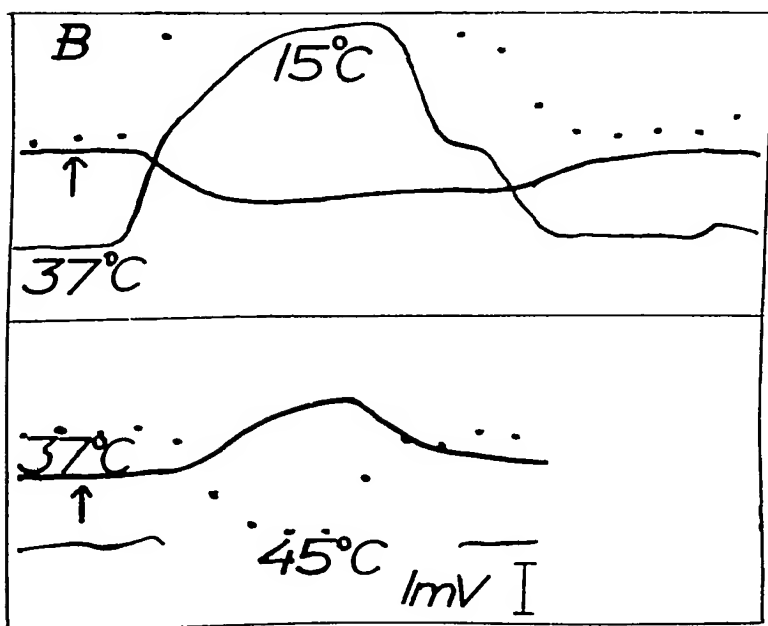
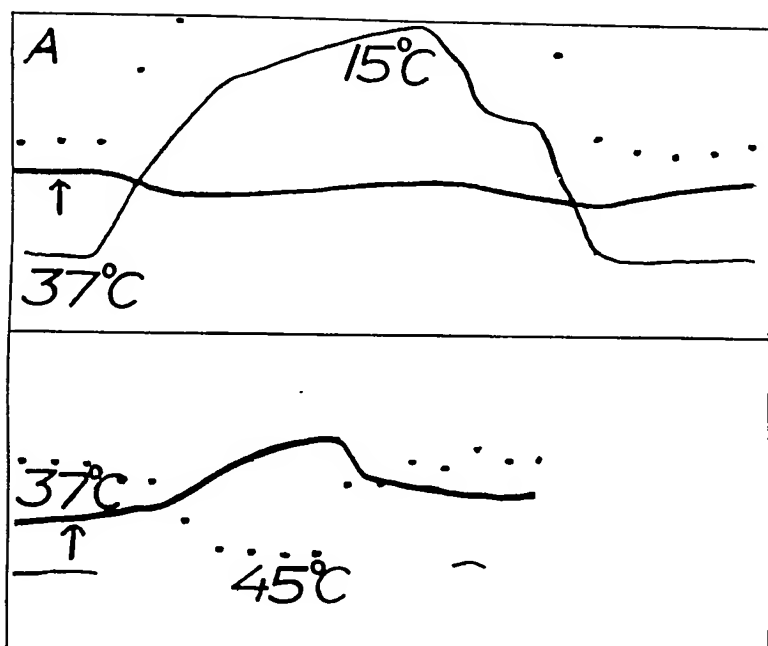


Fig. 17. Splenic nerve, cow. Response of membrane potential (arrow) to cooling 37°—15° and heating 37°—45°. Deflection upwards negativity. *A*: nerve which had been kept in normal Krebs solution. *B*: the same nerve treated during 30 minutes with potassium free Krebs. Marked as fig. 13.

1. 2. 3. 4. 5. 6. 7. 8. 9. 10.

1.

2.

3. 4. 5. 6. 7. 8. 9. 10.

1. 2. 3. 4. 5. 6. 7. 8. 9. 10.

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1. 2. 3. 4. 5. 6. 7. 8. 9. 10. 11. 12. 13. 14. 15. 16. 17. 18. 19. 20.

21. 22. 23. 24. 25. 26. 27. 28. 29. 30. 31. 32. 33. 34. 35. 36. 37. 38. 39. 40.

41. 42. 43. 44. 45. 46. 47. 48. 49. 50. 51. 52. 53. 54. 55. 56. 57. 58. 59. 60.

61. 62. 63. 64. 65. 66. 67. 68. 69. 70. 71. 72. 73. 74. 75. 76. 77. 78. 79. 80.

81. 82. 83. 84. 85. 86. 87. 88. 89. 90. 91. 92. 93. 94. 95. 96. 97. 98. 99. 100.

101. 102. 103. 104. 105. 106. 107. 108. 109. 110. 111. 112. 113. 114. 115. 116. 117. 118. 119. 120.

121. 122. 123. 124. 125. 126. 127. 128. 129. 130. 131. 132. 133. 134. 135. 136. 137. 138. 139. 140.

141. 142. 143. 144. 145. 146. 147. 148. 149. 150. 151. 152. 153. 154. 155. 156. 157. 158. 159. 160.

161. 162. 163. 164. 165. 166. 167. 168. 169. 170. 171. 172. 173. 174. 175. 176. 177. 178. 179. 180.

Chapter 2. The effect of variations in the potassium concentration on the thermal sensitivity of the action potential in A and C fibres

A fibres. The motor alpha fibres of the root are extremely sensitive to an increase of the potassium concentration of the surrounding medium. A conduction block is obtained already when the amount of potassium ions is about 2.5 times the normal one. Some roots are still more sensitive to K-ions, ceasing to conduct impulses when the potassium concentration of the medium has been doubled. These are nerves which also are abnormally sensitive to cooling, i. e. the spike optimum is at a higher temperature than normally and similarly the cold block.

If a normal root is washed with Krebs solution containing a 50 % increased concentration of KCl the negative afterpotentials disappear. But they may return if, after a period of cooling, the nerve again is warmed to 37°. This modest increase of the potassium concentration already leads to a diminution of spike height and the temperature sensitivity of the spike potential changes in such a fashion as to develop an optimum around 37°—40° and a higher blocking temperature to cooling than normally.

Fig. 19 is a plot of spike height against temperature in a root after 5 min. treatment with Krebs solution in which the potassium concentration had been raised by 100 %. The spike has its optimum height at around 40°—42° and the blocking temperature is at 20°. If the amount of potassium ions is further increased the optimum remains constant but the blocking temperature continues to move upwards with increasing potassium concentration until, at a K-ion increase of around 150 %, the spike is blocked already at 37°. If, at this stage, the nerve is warmed, the spike reappears and increases in magnitude up to 41°—43°.

An entirely different situation is encountered if the root preparation is replaced by a peripheral nerve with intact perineurial sheath. The phrenic or the peroneal nerve may be treated for an hour with a solution having a potassium concentration increased by 200 % without the appearance of other changes than a slight alteration in the temperature sensitivity of the spike and some diminution of the amplitude of the afterpotentials. It is

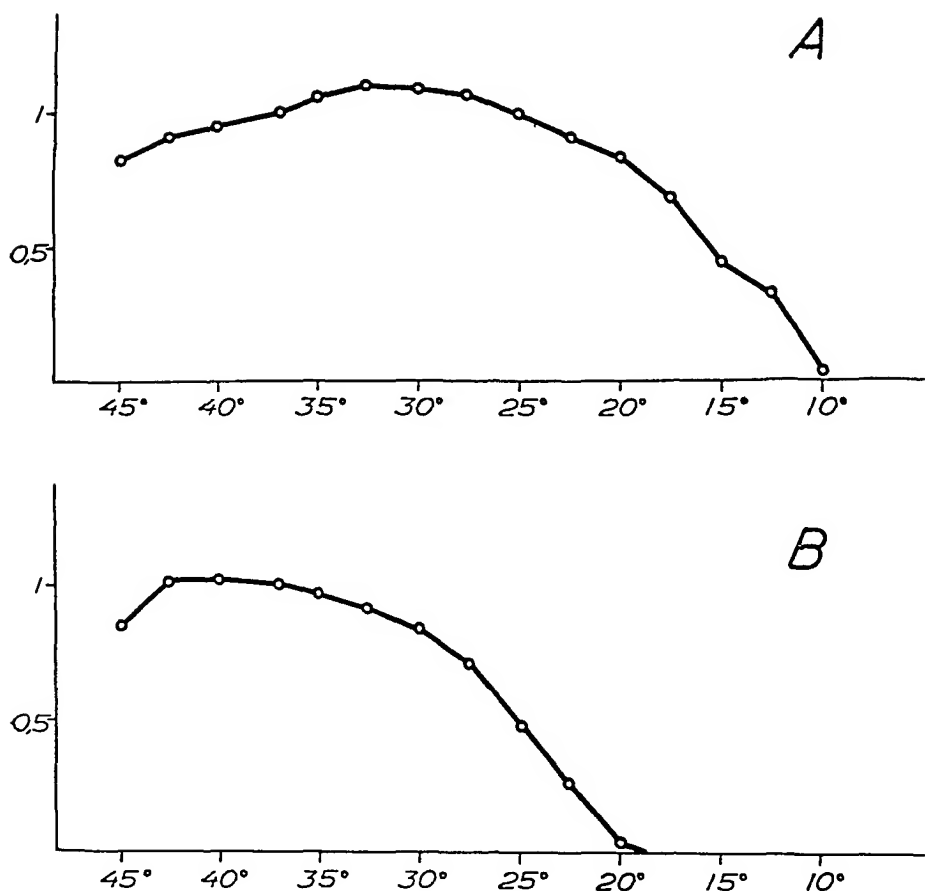


Fig. 19. 7th lumbar motor root, cat. Supramaximal stimulation of alpha fibres. Plot of spike height recorded at different temperatures in multiples of value at 37° in each experiment. *A*: root which had been kept in normal Krebs. *B*: the same root after 5 minutes treatment with Krebs containing twice the normal concentration of potassium.

difficult to explain this difference between roots and peripheral nerves but it does, indeed, seem likely that the perineural sheaths of peripheral nerves prevent the K-ions from influencing the nerve fibres.

If the motor root is treated with a potassium-free Krebs solution it becomes more resistant to cooling and this effect is noted both with the spike and the afterpotential. Fig. 20 shows how the thermal sensitivity is altered. Record A is a motor root with a relatively large negative afterpotential, maximal at 37°, which disappears at about 25°. After 10 min. treatment with potassium-

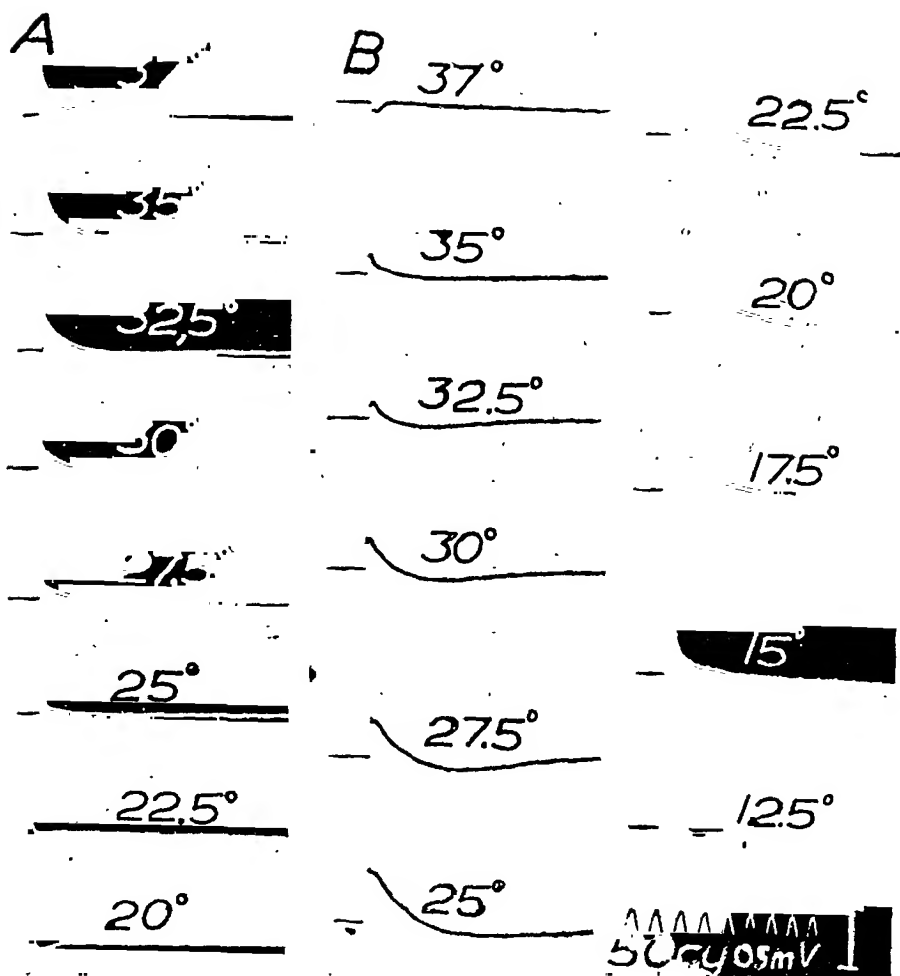


Fig. 20. 7th lumbar motor root, cat. Supramaximal stimulation of alpha fibres. Afterpotentials recorded at different temperatures. *A*: root which had been kept in normal Krebs. *B*: the same root treated 10 minutes with potassium free Krebs.

free Krebs solution (record B) the negative afterpotential at 37° has disappeared but it returns if the root is cooled and is then maximal at about 20—25°. It stands cooling to 15°. The figure also shows that the negative afterpotential in potassium-free Krebs is succeeded by a positive afterpotential, less marked in the normal root.

All experiments have shown this shift of the maximum of the negative afterpotential but the shift may be less well marked in

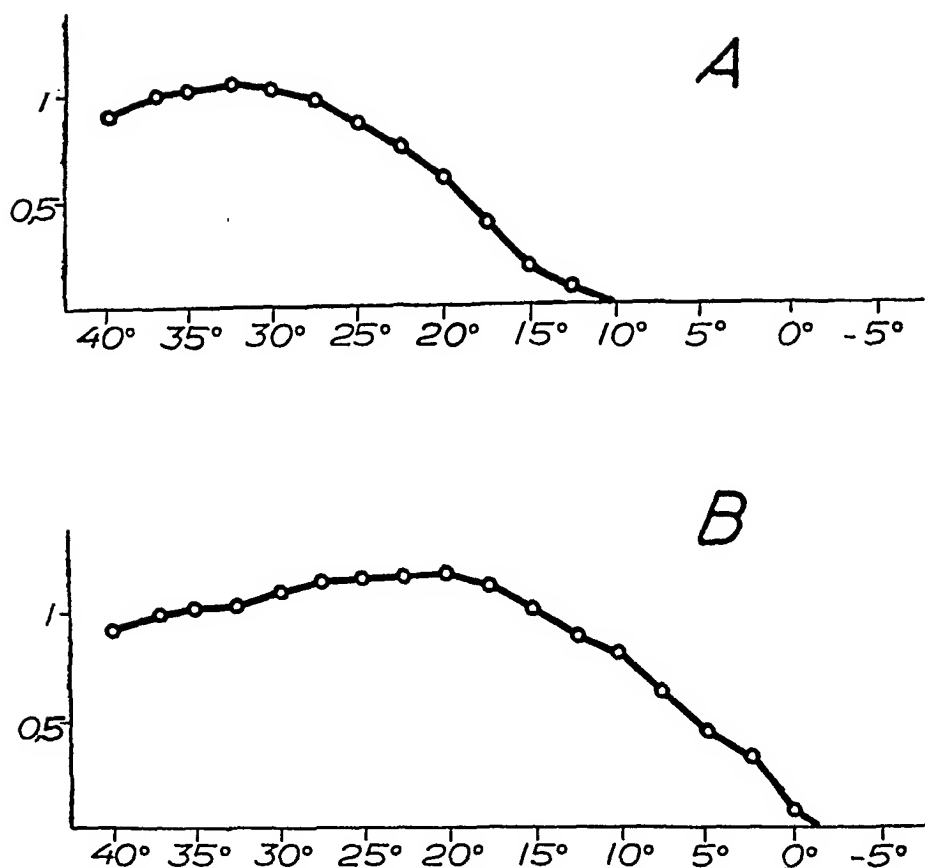


Fig. 21. 7th lumbar motor root, cat. Supramaximal stimulation of alpha fibres. Spike recorded at different temperatures in multiples of value at 37° in each experiment. *A*: root which had been kept in normal Krebs. *B*: the same root treated 10 minutes with potassium free Krebs solution.

some experiments than in the case illustrated in fig. 19. In roots from the cow the shift tends to be smaller.

Diagram B of fig. 21 shows how the spike height responds to potassium-free Krebs solution. The spike maximum shifts to 20° and further cooling is not capable of blocking conduction until a temperature as low as 0° has been reached. But there is also, during cooling, an interesting change of spike form. The spike potential increases in duration, the rising phase relatively little, the falling phase considerably, as shown by fig. 22 (record B). Thus, when cooling, the changing ratio between the duration of the ascending and descending phases of the potential makes the

spike in the potassium-free nerve (B) differ more and more from that in normal nerve (A).

It should again be emphasized that the potassium-free solution acts with a rapidity fully comparable with that of the solution containing an increased amount of potassium. Already after a couple of minutes one observes the shift of the blocking temperature characterizing the response of the potassium-free nerve to cooling.

C fibres. In comparison with the motor roots the C fibres are a great deal more resistant to potassium. For the conduction block it is necessary to use solutions in which the potassium concentration is 4—5 times the normal one. It was shown above that experiments with roots cannot straight away be compared with experiments on peripheral nerves without perineural sheaths. In order to compensate for this difference in the experimental conditions I made the test solution act for a longer time on the splenic nerve, generally for half an hour.

Fig. 23 shows the effect of an increased K-ion concentration on the temperature sensitivity of the spike. An increase of 100 % eliminates the large augmentation of spike height so characteristic for C fibres in response to cooling, and the maximum is reached around 25°. The spike diminishes again upon further lowering of the temperature. The block is at +5°. The diminution of spike height upon warming to 45° is also less prominent than in untreated nerve. The diagram illustrates also that with a potassium concentration of 200 % above normal the spike disappears already at 20° and that its maximum, normally at 5° for C fibres, has shifted to 37°—42°. As for the alpha spikes, so also in this case spike height is expressed in multiples of spike height at 37° for the particular experiment described. Therefore the identical spike heights at 37° do not imply that the spikes were of identical size in the three experiments summarized by the diagram. The reason for this procedure is that after each washing of the nerve that part of it which lies within the thermode is subjected to an alteration of the properties of the external conductor so that the spike height might be influenced independently of the specific effect of the medium tested. However, in

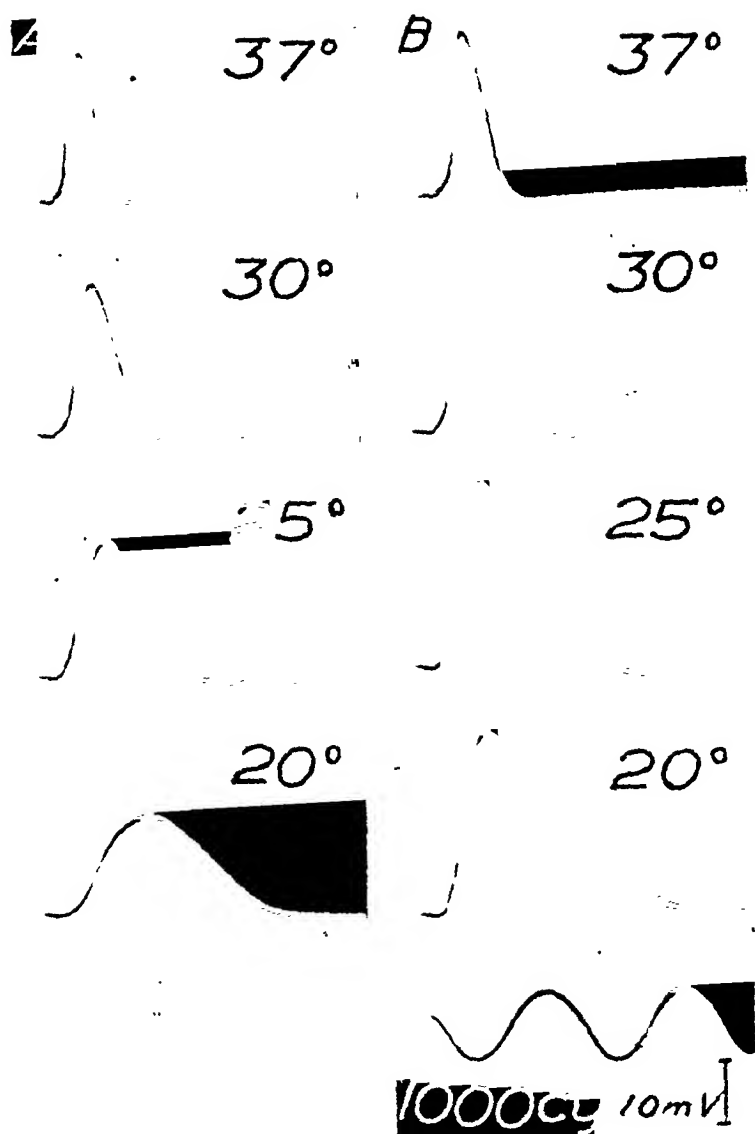


Fig. 22. 7th lumbar motor root, cat. Supramaximal stimulation of alpha fibres. *A*: root which had been kept in normal Krebs. *B*: the same root treated during 10 minutes with potassium free Krebs.

experiments designed with a view to maintaining the conditions as constant as possible I have never seen any diminution of the C fibre spike until the potassium concentration has been raised by more than 200 %.

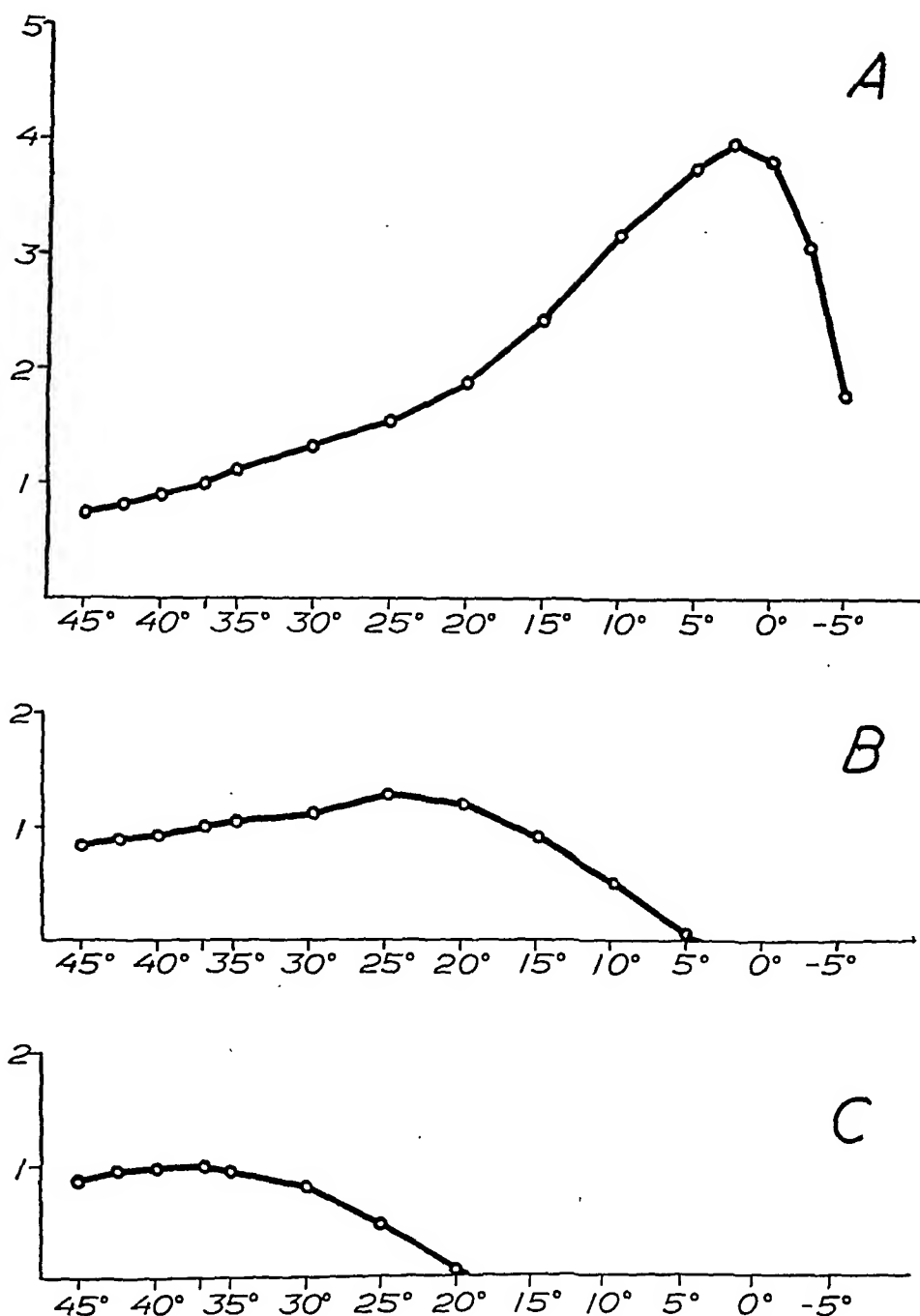


Fig. 23. Splenic nerve, cow. Supramaximal stimulation of C fibres. Spike height plotted against temperature in multiples of the value at 37° in each experiment. *A*: nerve which had been kept in normal Krebs. *B*: the same nerve after 30 minutes treatment with Krebs containing twice the normal amount of potassium. *C*: the same nerve treated 30 minutes in Krebs solution containing thrice the normal amount of potassium.

The effect of an increase of the potassium concentration of the solution upon the negative afterpotentials was also investigated with the splenic nerve. These diminished just as they did in A fibres but the C fibres were less sensitive in this respect than the latter. In many nerves the negative afterpotentials disappeared when the potassium concentration was raised by 100 % but in others a greater increase of the concentration of K-ions was necessary for their elimination.

The experiments aiming at determining the thermal sensitivity of the afterpotentials in potassium-treated nerves proved less satisfactory. In some nerves, particularly in those in which the negative afterpotentials disappeared at low potassium concentrations, it was noted that the thermal sensitivity of the negative afterpotentials remained the same until the afterpotentials disappeared for all temperatures. In other nerves, and generally in those in which the negative afterpotentials proved to be more resistant to potassium, their maximum was found shifted to a higher temperature level. As a rule this shift was insignificant, say, from the normal 25° to 30° , but the experiment illustrated in fig. 24 was one of some in which the maximum of the negative afterpotential after half an hour's treatment of the nerve with a solution containing potassium in a concentration of 100 % above normal, actually had shifted to 37° .

In experiments on the thermal sensitivity of the membrane potential in potassium-free Krebs solution the thermal equilibrium point (= maximum of membrane potential) regularly fell to a lower temperature level. It was expected that this would influence the thermal sensitivity of the negative afterpotential in potassium-free solution. Accordingly some nerves were tested with this object in mind but it was found impossible to demonstrate a shift of the maximum of the negative afterpotential to a lower temperature level. The spike, however, altered its capacity to withstand cooling. This effect was particularly evident in nerves in which the spike had its optimum at an abnormally high temperature but it appeared also in normally responding nerves. The large normal increase of spike height in response to cooling was further augmented by this treatment and the spike reached its maximum at a still lower temperature level.

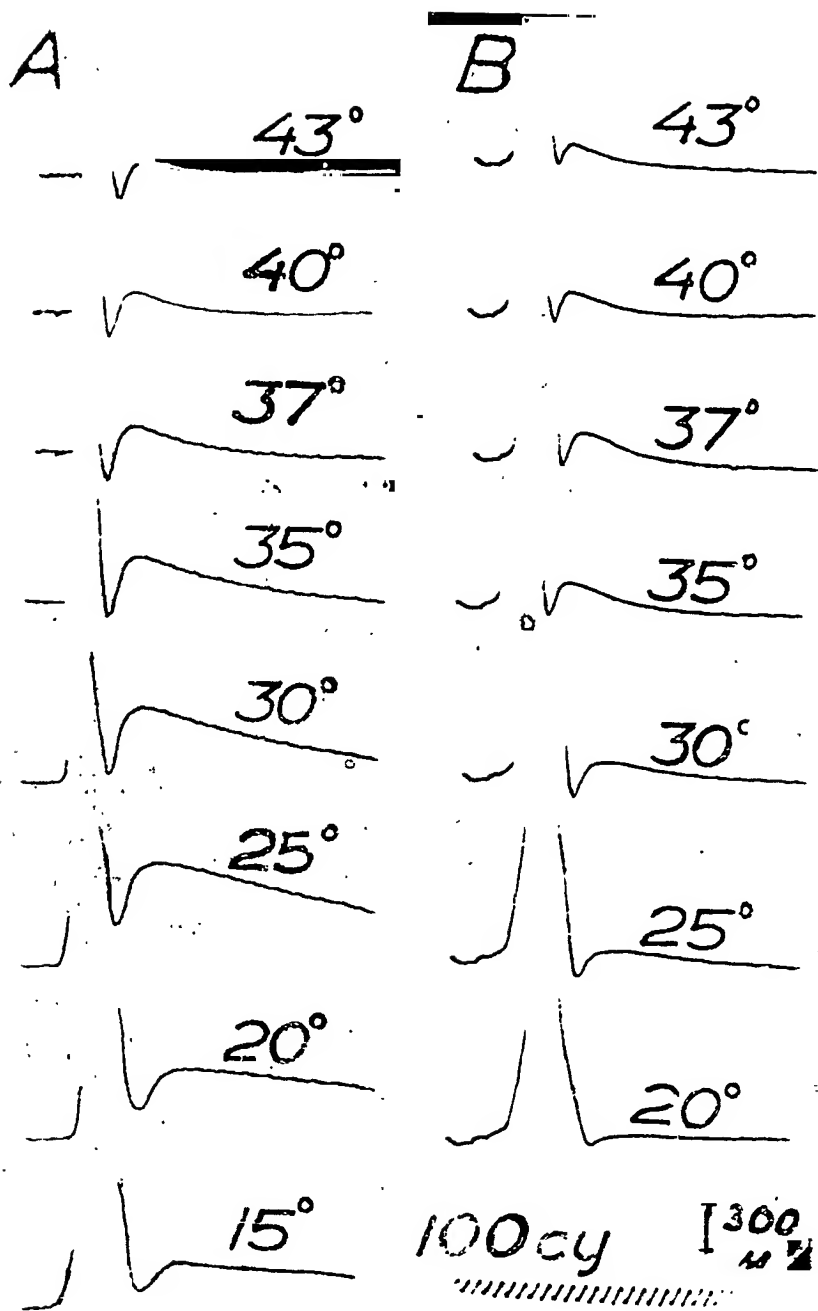


Fig. 24. Splenic nerve, cow. Maximal stimulation of C fibres. Afterpotentials recorded at different temperatures. *A*: nerve in normal Krebs. *B*: the same nerve after 30 minutes treatment with Krebs solution containing twice the normal amount of potassium.

Discussion

To begin with, let it once more be pointed out that the experiments have suggested that it is advantageous, in studying the effect of ion changes on the nerve, to use nerves lacking a sheath of connective tissue, preferably roots. The effect of potassium ions on peripheral nerve sets in at a very much slower rate and greater concentrations are required for making it measurable. It is highly improbable that this difference between the roots and the peripheral nerves could be wholly put down to a gradient of sensitivity along the axon even though such a factor might play a rôle. If the sheath is removed from a peripheral nerve the ions at once penetrate at a faster rate, to judge by the earlier appearance of the effect, and much smaller concentrations are required. Now this, when A fibres are concerned, might be ascribed to mechanical damage, to which the C fibres seem to be more resistant. But, on the other hand, the thermal sensitivity of the action potential is a delicate indicator of the degree of damage that a nerve has suffered and by this criterion several A fibres were perfectly normal after de-sheathing had been carried out. Therefore it does not seem likely that they had been damaged. The faster rate of penetration of the ions into the fibres themselves, after removal of the connective tissue sheath, provides a satisfactory explanation of the difference between roots and unsheathed nerves just as it does so for the difference between sheathed and de-sheathed nerves.

It is also interesting to note that a very small increase in the concentration of K-ions has marked effects on the mammalian nerves. The motor alpha fibres are blocked already by a potassium concentration 2.5 times that of the normal Krebs solution. The isolated fibres of the frog nerve require 8 times the normal concentration of the Ringer solution for the same effect (HERZ, 1946). It has also been found that the mammalian C fibres are far more resistant to potassium than the A fibres. This agrees with the recent findings of LORENTE DE NÓ (1947) on frog nerves. In agreement with earlier work on frog nerves is also the observation, made above, that excess potassium affects the afterpoten-

tial before it affects the spike (GRAHAM 1933, LORENTE DE NÓ 1947).

The effects of an altered potassium concentration of the external medium on membrane potential, spike potential and afterpotential suggest some correlations which deserve to be discussed. The following considerations are based on the observation by LORENTE DE NÓ (1947) that the magnitude of the negative afterpotential may serve as an index of the magnitude of the L-fraction of the membrane potential.

Excess potassium on a motor root is accompanied by the disappearance of the negative afterpotential. Accordingly the depolarization in response to cooling, obtained in A fibres, cannot have been due to a change in the L-fraction. Some other fraction of the membrane potential must have been involved. The method of measuring the membrane potential is, however, not so satisfactory from a strictly quantitative point of view as to warrant further conclusions from the fact that this depolarization to cooling is of the same order as that before treatment with excess potassium.

The loss of L-fraction in the roots treated with excess potassium cannot explain the shifts of the equilibrium point of the membrane potential to a temperature above 37° . The reason for this conclusion is the existence in such roots of a large positive response to warming from 37° . This positivity must be, as it were, a "new" phenomenon. It is too large to have occurred in normal nerve and to have been masked there by the depolarization of the L-fraction. The behaviour of the spike in the K-treated nerve supports this interpretation. The spike maximum, too, is shifted to a higher temperature level showing that other fractions than the L-fraction had been influenced by the increased concentration of K-ions.

The thermal response of the membrane of the A fibres in roots subjected to treatment with potassium-free Krebs solution may also be analyzed by comparing the thermal properties of the afterpotentials. By this criterion the L-fraction is certainly involved in the response to cooling. It is not necessary to assume the existence of contributions from other fractions. However, in the response to warming from 37° the depolarization obtained

can only to a very limited degree be due to depolarization of the L-fraction.

As to C fibres, the conclusion to be drawn from a comparison of afterpotentials with the changes of the membrane potential, after treatment with excess potassium, is that the depolarization in response to cooling is not at all or to a very small degree due to a depolarization of the L-fraction. This depolarization therefore engages other fractions of the membrane potential. In view of the general similarity between normal A fibres and C fibres treated with excess potassium it might be observed that in most cases the K-treated C fibres were lacking the negative afterpotentials seen in normal A fibres. The splenic nerve treated with potassium-free Krebs solution shows a shift of the equilibrium point of the membrane potential to a lower level but there is no corresponding shift in the thermal sensitivity of the negative afterpotential. This indicates that the changes in the behaviour of the membrane response to cooling might be due to changes in other fractions than L but further investigations are necessary for a final answer to this question.

In these experiments on the thermal sensitivity of the membrane potential the demarcation potentials have not been measured. No information is therefore available as to how the solutions tested change the absolute value of the membrane potential. But though information on this point would be valuable it is not indispensable for a discussion of the results. It is possible to explain the increase in the temperature necessary for the cold block in both the K-treated splenic nerve and the root by the assumption that the depolarizations due to potassium and cold are additive and so increase the total depolarization reducing the available margin of potential necessary for the spike. But the corresponding increase in membrane potential and spike optimum cannot be similarly explained, nor can the reduction in the thermal optima of the nerve in potassium-free Krebs be due to such factors. In both cases effects must have been obtained upon the primary processes underlying the thermal sensitivity of the functions themselves. They could not have been due to changes in the value of the membrane potential.

There is no reason to assume that the effects of the variations in the potassium concentration of the external medium have occurred in any other way than on account of the equivalent changes of the potassium concentration inside the fibres. This exchange of ions between external medium and inside of the fibres, be it in consequence of increased or decreased amount of potassium ions in the Krebs solution, must occur rapidly to judge by the fact that the thermal sensitivity of the nerve is changed already after a few minutes.

The fact that changes in the concentration of potassium ions can be employed to bring the thermal properties of A and C fibres to approach one another seems to be of great theoretical interest. A reduction of the internal concentration of potassium in motor A fibres makes the thermal sensitivity of the membrane potential and the negative afterpotential assume properties similar to those in C fibres, and, even if the spike potential of the A fibres does not become as resistant to cooling as that of the C fibres, it nevertheless becomes more resistant to cold than it was in normal nerve. Thus the change in the spike, too, is in the right direction. *Vice versa*, the C fibres can be made to assume thermal properties reminiscent of those of the A fibres by the opposite process, *i. e.* by increasing the internal concentration of potassium ions.

There are several ways in which to explain this tendency of A and C fibres to differ from one another with respect to their thermal properties as if this difference were due to corresponding differences in their sensitivity to potassium ions. The reason may, indeed, be that the C fibres cannot concentrate potassium against the same gradient as the A fibres so that their internal concentration of this ion actually remains lower than in the A fibres. On the other hand, the C fibres may be able to concentrate potassium but the mechanisms concerned in determining their thermosensitivity may be less sensitive to the potassium concentration than the corresponding processes in the A fibres.

However, it is necessary to realize that the potassium ions need not be the only agents that have to be considered in discussing these mechanisms. LACET and LUNDBERG (1948) have found that excess calcium on the motor root has the same effect on the

spike as potassium-free solutions although somewhat less pronounced. Apparently the K/Ca ratio is of importance in this as in so many other cases.

Taken as a whole the experiments have shown that the analysis of the thermal sensitivity of nerves provides valuable information about different fibre types. In addition the experiments have developed a general method for testing the effect of alterations in the external medium upon important nervous mechanisms.

A number of questions of general biological significance would also seem to be suggested by the striking effects of potassium on the thermal properties of nerve. It was pointed out in the historical section that there are reasons for assuming that the thermal sensitivity of nerves from poikilotherm animals varies with the temperature to which they have become acclimatized. Thus, for instance, several authors have noted that cold block occurs at higher temperatures in animals acclimatized to warmth than in those adapted to low temperatures. Similarly, there are results suggesting that the optimum of the A fibre spike bears some relation to the temperature to which the animals have become accustomed.

The present work raises the question as to whether such results may be due to variations in the potassium concentration (the K/Ca ratio). FENN, COBB, HEGNAUER and MARSH (1934) found a significantly lower value of potassium in nerves from November frogs than in those from March frogs. The temperature to which the animals were acclimatized is not mentioned in their work and so our question remains unanswered. Nevertheless results of this kind show that variations of the amount of potassium ions can and do occur in nerves. In this connexion it is interesting to note that the afterpotentials in A fibres from frog nerves vary in a characteristic fashion with the temperature to which the animals have been acclimatized (GASSER and ERLANGER, 1930) and that H. T. Graham has found the afterpotentials in potassium-treated frog nerves to be of the type normally found in summer frogs.

It was mentioned above that GASSER (1931) found the spike height of the frog nerve to diminish symmetrically in response to cooling from 30° whereas SCHOEPFLE and ERLANGER (1941) noted

that the spike increased asymmetrically by cooling, the falling phase lengthening out of proportion to the rising phase. Such discrepancies are less surprising when considering the results obtained in this work with variations of both temperature and potassium concentration. We recall that it proved possible, by diminishing the potassium concentration, to change both the temperature maximum of the spike as well as the relation between the durations of the falling and rising phases of the spike in response to cooling. But all such questions, related to the general problem as to the significance of the potassium concentration (or K/Ca ratio) for the adaptations to different temperatures have to be solved by further experimentation rather than by a comparison of historical data which merely can indicate that the problem merits investigation.

Summary

Part I

The effect of temperature changes on the membrane potential has been investigated in A fibres (motor roots, cat) and C fibres (splenic nerve, cow) of nerves kept in Krebs solution and locally heated or cooled by means of a thermode. The membrane potential of the A fibres was found to have its maximum value around 37° whereas in C fibres the maximum was around 25° . The maximum is defined as the point of thermal equilibrium because the membrane becomes negative relative to its value at the equilibrium point both when the nerve is warmed and when it is cooled.

The effects of temperature changes on spike and negative afterpotential were also investigated for A and C fibres (same preparations). The negative afterpotential in the A fibres had its maximum around 37° , in the C fibres around 25° . The spike potential in the A fibres reached its maximum height around 30° — 35° and the spike disappeared when the temperature fell to 7° — 15° . In the C fibres maximum spike height was around 5° — 10° and the spike did not disappear until the nerves had been cooled considerably below 0° . In autonomic C fibres of freshly excised cat nerves spike and afterpotential had the same thermal sensitivity as in the splenic nerve of the cow.

Thus, with respect to the functions investigated the A and C fibres proved to be equilibrated to different temperatures.

The relations between the changes in the membrane potential and the action potential are being discussed.

Part II

This part contains an analysis of how the concentration of potassium ions of the external medium influences the thermal sensitivity of the A and C fibres (same preparations as above).

Excess potassium shifts the thermal equilibrium point of the membrane potential of A fibres upwards, potassium-free solution downwards.

If a splenic nerve from which the sheath of connective tissue has been removed is subjected to the influence of excess potassium (twice or thrice the normal value of the Krebs solution) the membrane potential of the C fibres shifts its thermal equilibrium point to about 37° , i.e. to the value characteristic for normal A fibres. *Vice versa*, potassium-free solution shifts the equilibrium point of A fibres downwards to about 25° , i. e. to the value characterising normal C fibres.

Excess potassium blocks the motor alpha fibres already at a concentration of 2.5 times the normal one (Krebs solution). The C fibres require for the same effect an increase of 4—5 times the normal concentration.

The negative afterpotential disappears in the motor alpha fibres at a concentration of 1.5 times the normal one and the thermal sensitivity of the spike alters so that its height reaches its maximum at a higher temperature and the blocking temperature shifts upwards. An increase of the potassium concentration amounting to 2.5 times its normal value suffices to give the spike potential of the C fibres in the splenic nerve about the same thermal sensitivity as the normal motor alpha spike.

Treatment of the nerves with potassium-free Krebs solution not only lowers the equilibrium point of the membrane potential but also the temperature at which spike height is maximal. Thus, by this method, the maximum spike height of the motor alpha fibres may be shifted down to 20° , the blocking temperature down to 0° .

In the motor alpha fibres treated with potassium-free Krebs solution the maximum of the negative afterpotential also shifts downwards from the normal value around 37° to about 25° .

By treatment with excess potassium it has occasionally also been found possible to shift the maximum of the negative afterpotentials in C fibres (the splenic nerve) upwards.

All these effects of the variations of the potassium concentration are fully reversible.

In general it may be stated that, by variations in the concentration of potassium ions, the thermal properties of A fibres can be shifted in the direction of those of C fibres, and, *vice versa*, the thermal properties of C fibres in the direction of those of A fibres.

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STUDIES ON THE KINETICS
OF THE PARIETAL SECRETION
OF THE STOMACH

BY

K. J. ÖBRINK

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CHAPTER 1

The Purpose of the Present Investigation.

Investigations concerning the gastric secretion are numerous and many results have appeared since the fundamental works of BEAUMONT (1833).

The greatest interest has been directed towards the secretion of hydrochloric acid, but in spite of many attempts to solve the question of the formation of this acid in the stomach, the biochemical mechanism of this physiological process is quite unknown. Several theories have been suggested — cf. HARVEY & BENSLEY (1912), ZIMMERMANN (1925), HOLLANDER (1943), DAVENPORT (1939, 1940, 1941, 1942, 1943 a), DAVENPORT & FISCHER (1940), BULL & GRAY (1945), CONWAY & BRADY (1947), DAVIES, LONGMUIR & CRANE (1947) — but none of them has been supported by sufficiently convincing experiments.

If an explanation of the HCl-formation is ever to be arrived at, thorough *quantitative* analyses of the secreting procedure must be performed. Such analyses are relatively rare and often carried out with unsatisfactory methods. This treatise will, therefore, attempt to furnish further information about the quantitative aspects involved, i. e. *the kinetic interrelations between stimulation and its effects*.

Strong evidence has been given for the suggestion that the parietal cells are the elements of the gastric mucosa which are responsible for the HCl-formation. (Whether or not the acid is formed within the cells or on their surfaces is not known.) If the secretion of HCl is to be as nearly as possible uncomplicated, the parietal cells alone should be stimulated. The stimulus seems normally to arise from the pyloric mucosa — EDKINS (1906), KOMAROW (1938), UVNÄS (1942, 1943, 1945 a, b).

POPIELSKI (1920) discovered that histamine strongly stimulated the secretion of gastric acid. Since that time no better secretagogue acting exclusively on the parietal cells has appeared. In

fact most authors deny a non-parietal stimulation by the histamine — cf. BABKIN (1944).

In the present investigation histamine was therefore used as a stimulator of the gastric secretion. In order to depress the other cell-groups as much as possible the operative technique proposed by HEIDENHAIN (1878, 1879) was used on dogs, i. e. denervated gastric pouches were prepared. In this way the peptic secretion was thought to be partially depressed.

It would first be of interest to know the histamine concentrations which stimulate gastric secretion. This special problem will be discussed from a kinetic point of view.

The relation between the histamine concentration and its action on the parietal cells will be investigated and also the relationship between secretion rate and acidity, chloride concentration, excretion capacity of dyestuffs etc.

As a new substance has recently been found in the intestinal mucosa which inhibits the parietal secretion — *enterogastrone* — it was thought desirable to investigate its action on the secretion during histamine stimulation.

Attempts have also been made to investigate the quantitative relationship between the state of secretory activity and the electric potential difference across the gastric mucosa. These investigations, which have lent support to the theory that there is some relation between the secretion rate and the potential difference, will, however, be continued and published separately elsewhere.

A great number of papers having a connection with this work could not be mentioned, and the reader is therefore referred to the monographs of CARLSON (1923), ROSEMAN (1927) and BABKIN (1944). Concerning special histamine problems the monograph of FELDBERG & SCHILF (1930) may be consulted and on the question of the excretion of dyestuffs the books of MATSUO (1934—1935) are valuable.

CHAPTER 2.

Procedure.

The experiments in the present paper were carried out on animals, predominantly *dogs*. In some few cases *cats* were used.

It is thought most convenient to give a rather detailed description

of the procedure in the dog experiments. Changes in this procedure as well as the cat experiments will be described in the appropriate chapters.

Animals.

Dogs with vagotomized gastric pouches have been used for the investigations. The operations were carried out by Heidenhain's method (fig. 1). For technique see e. g. LE PLAY (1912). To avoid digestion

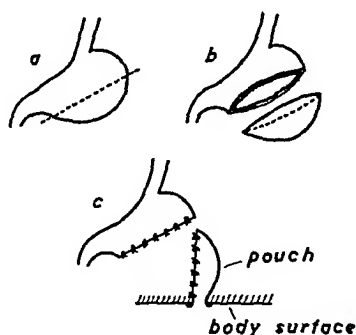


Fig. 1. *The Heidenhain pouch.* A schematic view of the operation.

of the surroundings of the "mouth" of the pouch, precautions were taken to prevent the gastric juice from flowing over the skin. This was achieved by using a Pezzer catheter (fig. 27, page 63) introduced through the opening of the pouch and allowed to remain there. The dogs usually very quickly became accustomed to this procedure which proved very effective in preventing digestion of the skin. It also had the advantage that a special measuring cylinder could be connected to this permanent rubber tube when desired.

The experiments were carried out on *fasting dogs*. *All possible care was taken to avoid psychic stimulation of the secretion.*

Before starting an experiment the pouch was usually washed out with warm saline, generally 20 ml. All the dogs were healthy and did not show any sign of discomfort during the performance of the present work.

For the present investigations 7 dogs were used. 3 of these in particular (dog S, R and B) were used for long periods (3—1 years). Two died from a prolapse of the pouch, two by an accident and one from distemper. Dogs S and B are still alive.

Stimulation of the gastric glands.

*Histamine dihydrochloride*¹ was used as a stimulating agent.

POPIELSKI (1920), KEETON, KOCH & LUCKHARDT (1920) and ROTH-LIN & GUNDLACH (1921) discovered that histamine (β -imidazolylethylamine) had a powerful stimulating effect on the gastric glands when given subcutaneously, but failed to stimulate the glands when injected intravenously in a massive dose. IVY & JAVOIS (1924) and GUTOWSKI (1924 a) when giving histamine intravenously over a period of 30 or 50 minutes, succeeded in getting a copious flow of gastric juice. They realized that not only the amount of histamine given, but also the *time* was of importance.

TEORELL (1932, 1933) was able to show mathematically that in Popielski's experiments the histamine was probably destroyed in the blood before causing any stimulation of the gastric glands. He proved that *a prolonged histamine concentration in the blood must be maintained if a stimulating effect is to be obtained.*

In order to balance the rate of the histamine destruction an appropriate amount of the drug must be administered by a *continuous intravenous injection*. The kinetics of the distribution of a drug administered in this way has been discussed by WIDMARK (1919), WIDMARK & TANDBERG (1924) and by TEORELL (1937 a, b). This stimulation technique for the gastric glands has been used by only a few authors: IVY & JAVOIS (1924), GUTOWSKI (1924 a), TEORELL (1933), ENGSTRÖM (1935), EMMELIN, KAHLSON & WICKSELL (1941), BJÖRKMAN, NORDÉN & UVNÄS (1943), EMMELIN (1945), McELIN & HORTON (1946) and LINDE, TEORELL & ÖBRINK (1947).

Technique of injection.

In some experiments the histamine was given *subcutaneously* but the intravenous route was generally used.

The *continuous intravenous injection* was performed by using a *motor driven syringe*. At the beginning, an apparatus described by LINDGREN (1943) was used, but later on a new device, which has proved very suitable, was adopted.²

¹ In this paper all figures are expressed in terms of *histamine-dihydrochloride*. To transform these to histamine base or histamine diphosphate the figures must be multiplied by 0.60 and 1.67 respectively.

² The technical work and the details of construction were executed by Mr. S. LILIEDAHL.

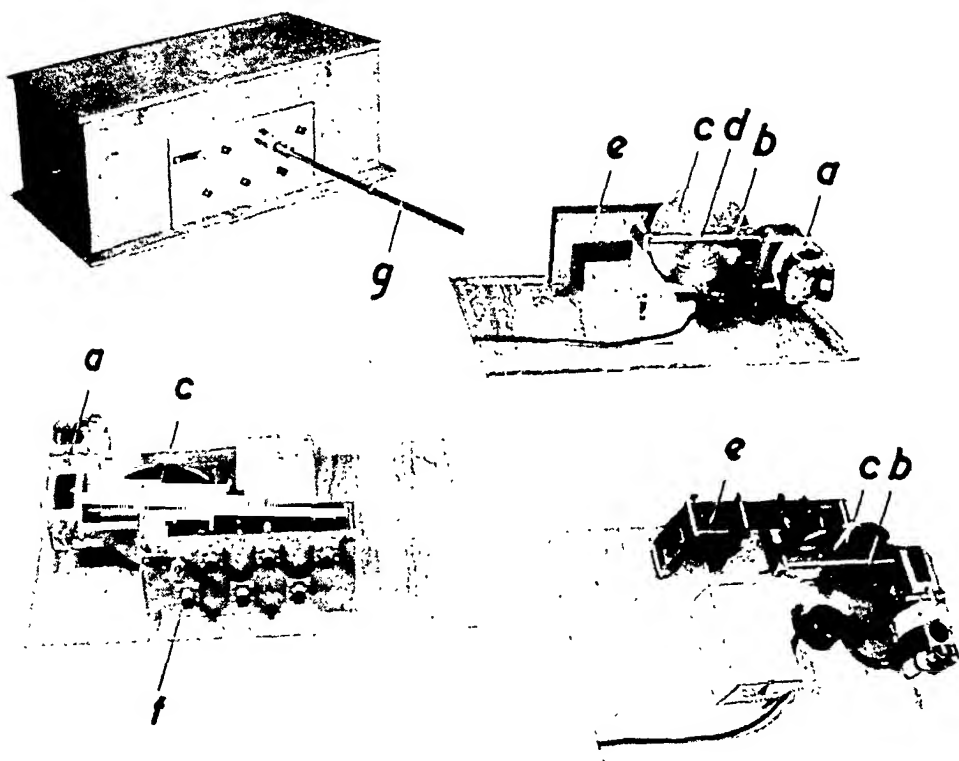


Fig. 2. *The injection apparatus (Motor part).* *a* is the electric motor, which drives the small wheel *b*. This acts on the large wheel *c*. *b* can be moved along the axle *d*. The speed is lowered in the gear box *e* 5 times between each connection *f* for the spindle *g*, which leads to the syringe holder in fig. 3.

As such an apparatus may often be useful in other laboratories a short description will be given here.

The injection apparatus.

The motor part (fig. 2).

The electric motor (*a*) is an ordinary gramophone motor (but of course any motor that has a very constant speed may be used). The motor drives a small wheel (*b*) 28 mm in diameter, the peripheral part of which is made of rubber and pressed against a larger wheel (*c*) 150 mm in diameter. The axles of the two wheels are at right angles to each other. By adjusting the small wheel nearer to or further from the center of the big one, the speed of the latter may be made greater or less. For this purpose the small wheel can be moved along an axle (*d*) graduated from 1 to 6, where 1 corresponds to the periphery of the big wheel and 6 to the center. It is not

necessary nor convenient to place the rubber-wheel nearer the center than at 5. Thus by moving the small wheel from 1 to 5, the speed can be increased 5 times.

From the large wheel an axle leads to a gear box (e). This lowers the rate in steps of 5 times. The lowest rate is 5^{-5} times the rate of the big wheel. By altering the position of the little wheel and by using different connections (f) of the gear box the speed of movement derived

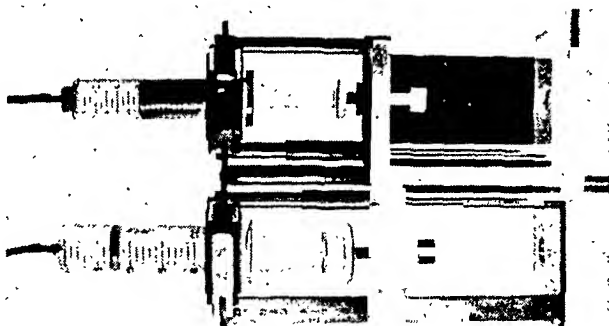


Fig. 3. *The injection apparatus (Syringe holder)*. This is only a modification of the one constructed by LINDGREN (1943).

from the machine may be changed by a factor as much as 5^6 . The lowest speed is about 0.4 turns/hour and the highest about 100 turns/minute. From the gear box a connecting spindle (g) leads to the syringe holder.

The syringe holder (fig. 3).

The principal part of this is a screw, which is driven by the spindle. The screw drives two cross-pieces which are in contact with the syringes. The mechanism of this part is the same as described by LINDGREN (1943). The only difference is that two syringes can be driven at the same time instead of one. If the height of the screw-thread is 1 mm, that distance corresponds to one turn of the spindle.

In order to determine the position of the little wheel and the spindle, the diagram in *fig. 4* can be used. If a particular speed for the syringe is desired this must be transformed into turns/minute of the spindle. 100 times the reciprocal of that value may be looked up in the diagram. The column in which this quotient is found indicates the connection point for the spindle and the horizontal intercept to the crossing point between a horizontal line from the number and the oblique line in the diagram indicates the position of the small wheel (graduations on the axle).

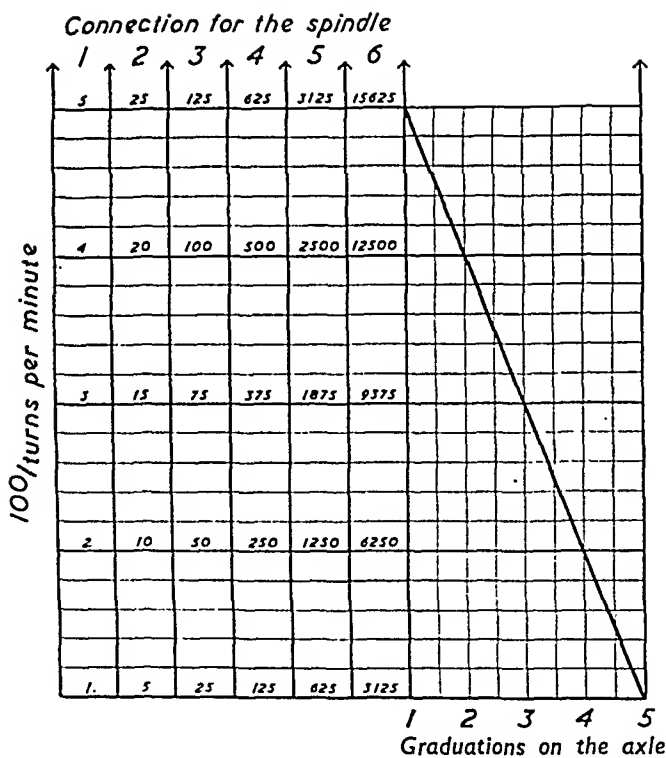


Fig. 4. Diagram for the use of the injection apparatus. For description see text! (Example: The injection rate wanted needs 2 turns per minute by the screw of the syringe holder. 100/turns per minute — the ordinate in the figure — is $100/2 = 50$. This number is found in column 3, which means that the spindle must be connected to the third connection. The horizontal from 50 crosses the oblique line at the graduation 4, which indicates where the small wheel shall be placed on the large one.)

The contents of the syringe are pressed through a narrow rubber tube to a needle of the shape in *fig. 5*. By using this type of needle the rubber tube can be connected to the one mouthpiece, while blood can be obtained through the other one, thus indicating a perfect position in the vein. The needle is fixed with adhesive tape.

Method of collecting the gastric juice.

A special measure is seen in *fig. 6*. It consists of a measuring cylinder supplied with a stopcock with a very wide hole. This makes it possible to collect the juice for a desired time and to empty the measure instantaneously even if the content should have a high viscosity. A suction pump can be connected to a side-pipe, if desired. A negative pressure of 30 cm of water is then convenient. The tube, which is graduated in $1/5$ of a ml, is joined to the Pezzer catheter during the experiment.

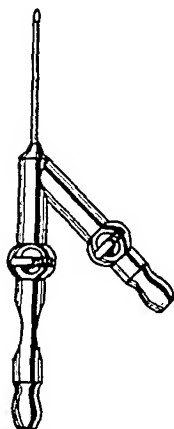


Fig. 5. *The injection needle.*

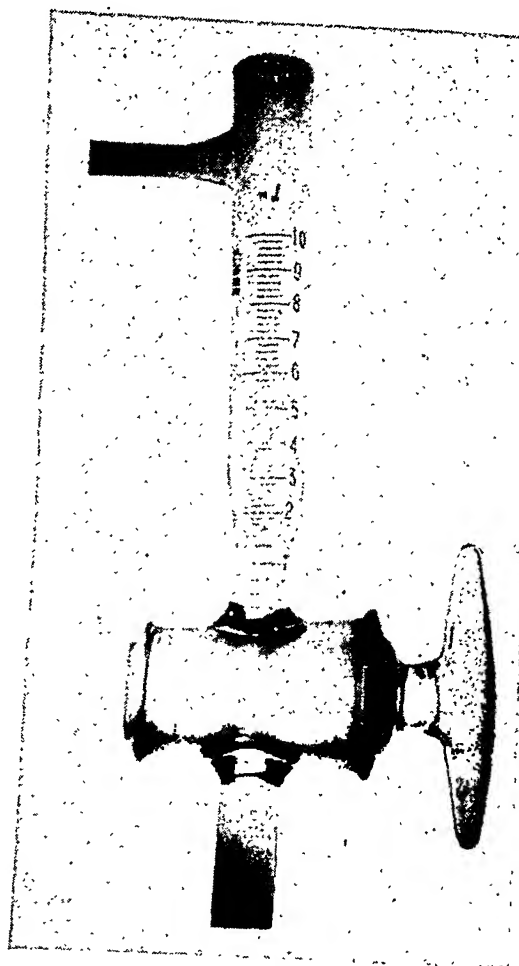


Fig. 6. *The measuring cylinder for collecting the gastric juice.*

By the technique described in this chapter it is found possible to record the secretion volumes at very short intervals. *2 minutes was usually the time used for determining the secretion rates whereas samples for analyses were taken every 10 minutes.*

CHAPTER 3.

Methods of Analysis.

All the analytical procedures are collected in this chapter. They are described in the following order and include the determinations of

- | | |
|---------------------|--------------------------|
| 1. <i>pH</i> | 7. <i>reducing power</i> |
| 2. <i>acidity</i> | 8. <i>calcium</i> |
| 3. <i>chloride</i> | 9. <i>bicarbonate</i> |
| 4. <i>histamine</i> | 10. <i>phenol red</i> |
| 5. <i>pepsin</i> | 11. <i>neutral red</i> |
| 6. <i>nitrogen</i> | |

The *statistical errors of the estimations* are given in connection with the description of the methods.

They are expressed as the errors of *single determinations* and were calculated as the standard deviation from series of 20 separate determinations on identical objects. If "blanks" and/or "standards" are included in the analytical procedure, their errors are also considered.

In some methods (2, 3 and 8) the result (x) was calculated from a "standard" (st) with its error σ_{st} and a "sample" (sa) with its error σ_{sa} as

$$x = \frac{sa}{st} \cdot C \quad (1)$$

(C is a constant).

The error of a single determination was then calculated as

$$\sigma_x = \pm C \cdot \frac{\sqrt{st^2 \sigma_{sa}^2 + sa^2 \sigma_{st}^2}}{st^2} \quad (2)$$

In some methods (6, 7 and 9) the result (x) was calculated from a "blank" (bl) with its error σ_{bl} and a "sample" as

$$x = (bl - sa) \cdot C \quad (3)$$

The error of a single determination was then calculated as

$$\sigma_x = \pm C \cdot \sqrt{\sigma_{bl}^2 + \sigma_{sa}^2} \quad (4)$$

1. pH determinations.

pH was determined by aid of a glass electrode arrangement. Accuracy of reading ± 0.05 pH units.

2. Acidity determinations.

a) Total acidity:

The volumes available for the acidity determinations varied from some mls down to the order of 0.1 ml. A microtitration on 0.1 ml juice mixed up with 4 ml H_2O was therefore performed with 0.01 N NaOH using *brom-thymol-blue* as an indicator (pH-range 6.0—7.6). The strength of the NaOH solution was checked every day using carefully controlled 0.1 N HCl (standard). The same pipette was used for the standard and the samples. Using this procedure errors due to the pipette, indicator, and CO_2 admixture were compensated. The neutralization of NaOH by 4 ml H_2O was determined as a blank. (This was in most cases in the order of 0.00—0.01 ml. The error of the blank determination will have an insignificant influence on the error of the method.)

b) Free acidity:

Free acidity was determined either by an electrometric titration (pH 3.5) or by the use of Töpfer's indicator (di-methyl-amino-azo-benzene, endpoint pH 3.5). The volume used for these titrations was 1.0—5.0 ml.

With the histamine technique used in the present paper the difference between the free and the total acidity was very small. For this and other reasons (cf. page 52) only the total acidity values are discussed in the following.

The error of the total acidity determinations (valid for the total range in this paper): $= \pm 2 \text{ mN}^1$.

3. Chloride determinations.

a) in gastric juice:

The chloride titrations were performed on the same samples as were used for the total acidity determinations.

2 ml 0.1 N H_2SO_4 were added and then the samples were titrated electrometrically with 0.005 N AgNO_3 .

b) in blood plasma:

Heparinised blood was centrifuged. 0.1 ml plasma + 4 ml H_2O + 2 ml 0.1 N H_2SO_4 were titrated in the same way as the gastric chlorides. The proteins do not affect the result.

Standard and blank titrations were performed as in the acidity determinations.

The error of the method varied from time to time according to the conditions of the measuring instruments. Under bad conditions it was $\pm 6 \text{ mN}$ but all the data referred to in this paper have the smaller error given below.

The error of the chloride determinations (valid for the total range in this paper): $\pm 2 \text{ mN}^1$.

4. Histamine determinations.

The method described by CODE (1937) was used. The determinations were performed on 4—5 ml plasma from heparinised blood. The concentration-action curve of histamine on the guinea pig's small intestine was determined and is seen in fig. 7 — cf. EMMELIN, KAHLSON & WICKSELL (1941). Our curve, like that of the authors referred to, demonstrates the necessity of carrying out the investigations with concentrations of histamine which cause only a submaximal contraction (the rising part of the curve). All histamine values are expressed as histamine dihydrochloride (cf. page 10).

The error of the histamine determinations in blood plasma: $\pm 3.7 \mu\text{g}/100 \text{ ml}$ $= 5\%$ of the mean value.

5. Pepsin determinations.

Pepsin determinations were carried out according to the method described by RIGGS & STADIE (1943). This method consists of a turbidimetric analysis of a protein solution, which was digested for 5 minutes. The digestive power is expressed in mg protein digested per ml gastric juice during 1 minute.

The error of the pepsin determinations: $\pm 0.005 \text{ mg protein/ml/min.} = 1.8\%$ of the mean value.

¹ mN = millinormality = milliequivalents per liter.

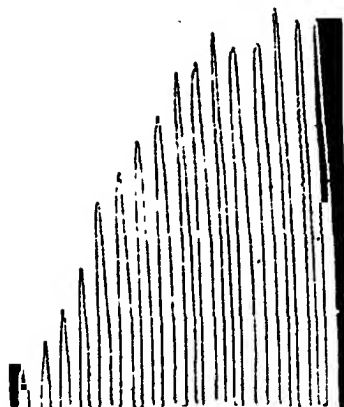


Fig. 7. *Concentration-action curve* of histamine action on a guinea pig's small intestine. The contractions correspond to the histamine-doses: 0.005, 0.010, 0.015, 0.020 etc. μg histamine dihydrochloride per ml bath solution. The volume of the bath = 10 ml.

6. *Nitrogen determinations.*

1.0 ml gastric juice was combusted with 1 ml concentrated H_2SO_4 for 5 hours. 2—3 drops of H_2O_2 were added and the combustion continued another 2 hours. The determinations were then performed by the method of TEORELL (1928).

The error of the nitrogen determinations (valid for the total range in this paper): ± 4.0 mg N/100 ml.

7. *Determinations of reducing power.*

2 ml of gastric juice + 5 ml of a 10 per cent HCl were heated in a boiling water bath for 30 minutes. After cooling they were neutralized with 30 per cent NaOH. The reducing power of the remaining volume and of another 2 ml juice were then determined — cf. IHRE (1938).

This was done by the method of SCHALES & SCHALES (1945) with the modification that a sodium-cyanide-carbonate solution was used instead of a phosphate buffer solution. This modification which was mentioned by SCHALES and SCHALES was adopted from the paper of FOLIN & MALMROS (1929).

The difference in the reducing power of the hydrolyzed and the non-hydrolyzed sample was compared with that of known solutions of glucose and expressed in mg glucose per 100 ml.

The error of the reducing power determinations (valid for the total range in this paper): ± 8 mg/100 ml.

8. *Calcium determinations.*

The oxidimetric method with ceric sulphate as described by NIELSEN (1941, p. 190) was used.

The error of the calcium determinations (valid for the total range in this paper): ± 0.45 mg/100 ml.

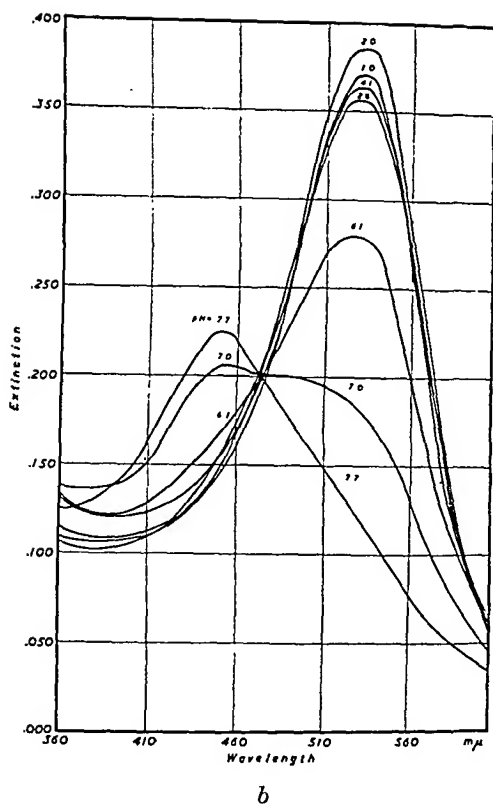
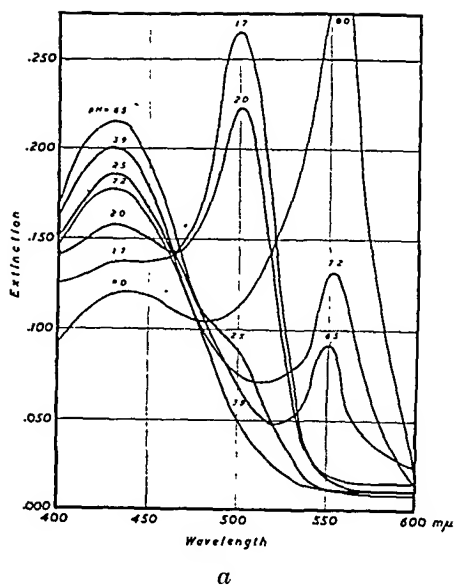


Fig. 8. Absorption curves of phenol red (a) and neutral red (b) at different pH:s.

9. Bicarbonate determinations.

The bicarbonate concentration in glycine solutions was determined according to the method of WEST, CHRISTENSEN & RINEHART (1940).

The error of the bicarbonate determinations (valid for the total range in this paper): ± 2.8 mN.

10. Phenol red determinations.

The concentration of phenol red was determined in the estimations of the size of the "lake," which is a residual volume of the contents in the gastric pouch (cf. page 63).

Some exact volume, a ml, of phenol red was instilled into the pouch cavity and mixed up with the "lake" contents as far as possible. The phenol red solution was then allowed to leave the pouch.

The colour of the dye was determined before (C_a) and after (C_x) the instillation. The size of the "lake" (x) was then easily found from

$$x = \frac{a(C_a - C_x)}{C_x} \quad (5)$$

This gave a minimal value of the "lake" because there was no guarantee that a complete mixing had occurred.

In order not to let changes in pH influence the colour determinations in a Coleman electrophotometer, these were carried out at the *isosbestic point* (wave length 463 m μ). This was found by determining the absorption curves of the same concentration of phenol red at several different pH:s in a Beckman spectrophotometer. The absorption curves are seen in *fig. 8 a*.

The error of the phenol red determinations: $\pm 0.6\%$ of the mean value.

11. *Neutral red (N. R.) determinations.*

N. R., like phenol red, was determined in a Coleman electrophotometer at the *isosbestic point* (wave length 472 m μ).

The absorption curves from which the isosbestic point is obtained are seen in *fig. 8 b*.

The error of the neutral red determinations: $\pm 0.6\%$ of the mean value.

CHAPTER 4

The Histamine Distribution in the Body.

Theoretical considerations.

Introduction.

It is well known that the concentration in blood and tissue of a drug given subcutaneously or intravenously will follow different *time-concentration curves*. It is easy to understand that a very high concentration in the blood will occur immediately after a rapid intravenous injection, whereas a subcutaneous injection will cause a slower increase. The *kinetics* (i. e. the time relations of the drug concentration) will depend on the drug itself and the possibility of its passing from one phase to another and the forces causing this passage. Reactions of the drug, such as combination or destruction, will also influence the concentration of the active drug. In the case of an intravenous injection the *injection rate will be of importance*.

Very little study has been devoted to this problem. TEORELL (1937 a, b) has tried to develop a general mathematical view of the problem. In his papers a short review of the earlier literature in this field is given. It is therefore unnecessary to repeat it here. It is, however, necessary to give a short summary of Teorell's results, in order to make it possible to discuss some problems in our own work.

Teorell's work.

The validity of the mathematical formulae given is limited to electrically uncharged substances, i. e. strong electrolytes do not behave in the same manner as undissociated molecules because they are also influenced by electrical potential gradients. If the concentration gradient is the only driving force, the amount passing a boundary will follow Fick's law for unidimensional molecular diffusion:

$$\text{Amount} = \text{diffusion coeff.} \times \text{conc. gradient} \times \text{surface} \times \text{time.}$$

If the diffusion coeff. and surface are taken together in a "permeability coefficient" as k'_n the formula may be rewritten.

$$\begin{aligned} \text{Amount across boundary in the time unit} &= \\ &= \text{permeability coeff.} \times \text{concentration difference.} \end{aligned}$$

A concentration is, however, the amount divided by the appropriate volume, and so we may take the permeability coefficient together with the distribution volume, which can be considered as constant, and substitute them with a "velocity constant" k_n . The temperature is supposed to be constant.

The velocity constants involved are:

| | | | |
|-------|-------------------------|---------------|--|
| k_1 | for the diffusion depot | \rightarrow | blood (extravascular administration only) |
| k_2 | » | » | blood \rightarrow tissue |
| k_3 | » | » | tissue \rightarrow blood |
| k_4 | » | » | blood \rightarrow kidneys or lungs (or blood inactivation) |
| k_5 | » | » | tissue inactivation. |

A. The subcutaneous case.

If x , y and z denotes the amount of the drug in *depot*, *blood* and *tissue* respectively, and N_0 = the initial dose given to the depot, the following differential equations may be derived:

for the blood:

$$\frac{dy}{dt} = k_1x - k_4y - (k_2y - k_3z) \quad (6)$$

for the tissue:

$$\frac{dz}{dt} = (k_2y - k_3z) - k_5z \quad (7)$$

By combining the two equations it is possible to solve for y and z :

$$y = C_1e^{m_1t} + C_2e^{m_2t} + Se^{-k_1t} \quad (8)$$

$$z = R_1e^{m_1t} + R_2e^{m_2t} + He^{-k_1t} \quad (9)$$

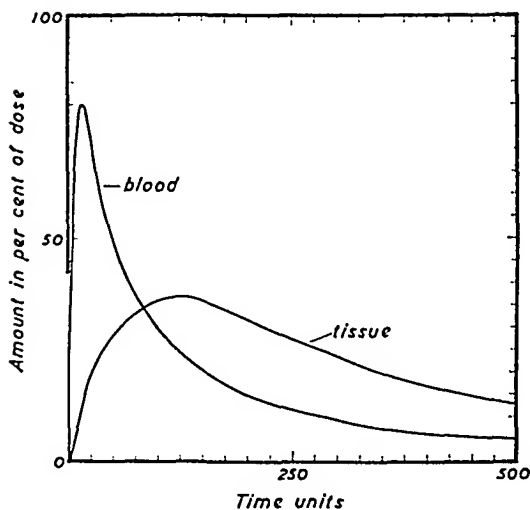


Fig. 9. The distribution of a drug injected subcutaneously. See eqs (8) and (9). [$k_1 = 0.2$; $k_2 = 0.01$; $k_3 = 0.005$; $k_4 = 0.005$; $k_5 = 0.002$.] — After TEORELL (1937 a).

where the new symbols C_1 , C_2 , S , R_1 , R_2 , H , m_1 and m_2 are all constants and functions of the velocity constants k_1 — k_5 and the dose N_0 .

The relations (8) and (9) are seen in fig. 9, where the amounts in per cent of the initial dose N_0 are plotted against time.

B. The continuous intravenous case.

In the case of a *continuous intravenous injection* the rate of “resorption” will be substituted by the injection rate, denoted by r . Thus in equation (6), k_1x may be substituted by r . The rest of the system remains unchanged. The final mathematical solution can then be written:

$$y = r \cdot [A_1 e^{m_1 t} + A_2 e^{m_2 t} + I] \quad (10)$$

and

$$z = r \cdot [B_1 e^{m_1 t} + B_2 e^{m_2 t} + J] \quad (11)$$

where A_1 , A_2 , I , B_1 , B_2 and J are constants and functions of the velocity constants k_2 — k_5 . The rest of the symbols retain their previous significance.

Fig. 10 gives the graphical representation of the equations (10) and (11). It must be noted that both the blood and tissue curves approach a constant value, which can be mathematically expressed by putting time $t = \infty$.

Then (10) and (11) are reduced to:

$$y_\infty = r \cdot \frac{k_3 + k_5}{q} \quad (12)$$

and

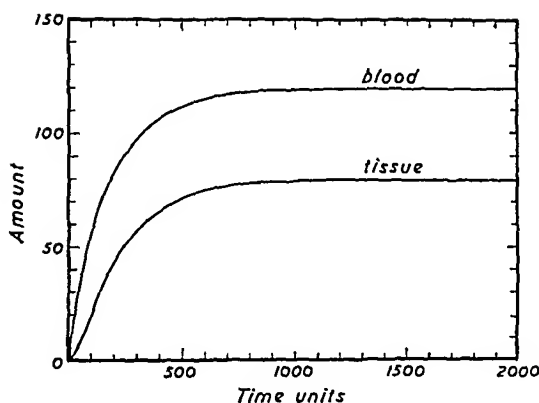


Fig. 10. *The distribution of a drug after continuous intravenous injection.* See eqs (10) and (11). [$r = 1$; $k_2 = 0.01$; $k_4 = 0.005$; $k_5 = 0.005$.] — After TEORELL (1937 b).

$$z_{\infty} = r \cdot \frac{k_2}{q} \quad (13)$$

where $q = k_2k_5 + k_3k_4 + k_4k_5$.

The above is a short review of those parts of the work of TEORELL, which have an application to the present paper. Starting from these equations some other relations can be investigated:

The author's calculations.

If the interest is directed for a moment to the *total amount* of a drug accumulated in the blood and tissue after a subcutaneous injection during the whole period of an experiment, the following will be found:

The *total amount in the blood* is represented by the area included in the curve given in eq. (8). This area is

$$Y = \int_{t=0}^{t=\infty} y \, dt \quad (14)$$

where y has the same significance as in eq. (8). Integration of (14) gives

$$Y = \frac{k_3 + k_5}{q} \cdot N_0 \quad (15)$$

In an analogous way the *total amount in the tissue* will be found by integrating the eq. (9) and the result is

$$Z = \frac{k_2}{q} \cdot N_0 \quad (16)$$

The first point of interest is that k_1 has disappeared from these formulae, i. e. *after a subcutaneous injection the total amount of a drug*

found in blood or tissue is independent of kind or rate of resorption. It depends only upon the dose given in the depot.

On the contrary the *maximal value* obtained in blood or tissue during an experiment after a subcutaneous injection will not be independent of k_1 , as may easily be shown. The general expressions, however, are too complicated to be solved here. (TEORELL has proved it for blood in a special case ($k_2 = k_3 = k_5 = 0$).)

The integrations for (15) and (16) were made from $t = 0$ to $t = \infty$. In practice the result of integration will be just the same if it is made between $t = 0$ and $t =$ some short measurable time (e. g. 1—3 hours) when we are dealing with drugs, that are quickly eliminated or destroyed as histamine.

The second point of interest is the similarity that exists between the eqs (12), (13) and (15), (16).

The consequences of these expressions will be discussed below (chapter 7, page 37).

Experimental data.

Earlier experiments.

There have been many observations after continuous intravenous injections of substances which support the theoretical considerations given above. The concentration of any substance injected in this way will undoubtedly approach a constant level. Both non-electrolytes and electrolytes may be taken as examples. See WIDMARK (1919) for acetone and WILHELMJ & MOSKOWITZ (1932) for uric acid.

The behaviour of histamine itself has also been investigated — EMMELIN, KAHLSON & WICKSELL (1941), EMMELIN (1945). The main thing is, however, to prove that the steady state of concentration is proportional to the injection rate (according to eq. 12 and 13). This has not been done to the author's knowledge. The studies of EMMELIN & KAHLSON (1944) have been restricted to correlating the injection rate and the concentration of histamine in the gastric juice, and their results corroborate the theoretical propositions.

The author's experiments.

The method used for the histamine determinations was the one proposed by CODE (1937). (Cf. page 16.)

Analyses were made in the venous blood from a leg before the start of the experiment, "*base value*," and when the injection of histamine had caused a steady state of the secretion rate, "*steady state value*."

The increment caused by the histamine injection is the "*steady state value*" minus the "*base value*."

In some series of experiments the analyses were performed on *whole blood* but gave no regular covariation of the histamine increment and the injection rate of histamine.

In the experiments described below the *plasma* was analyzed. The injection rates were changed at random. The rates of injection were usually less than 8 ml/hour.

In every experiment there was a considerable "*base value*," which did not cause any gastric secretion. If the method of analysis was specific for histamine it means that histamine was present in the blood plasma in an inactive form. This question will be discussed below.

The main question is to determine the possible covariation between the rate of injection of histamine and the steady state increment. *Fig. 11* shows this relation (dog S), and the experimental values are correlated to the eq. (12). The coefficient of correlation is 0.94. The other statistical data are seen in the legend to the figure.

The filled circles in the figure denote the corresponding secretion rates. They indicate that most of the analyses were performed during *supramaximal* stimulation. This is essential as it shows that the linear covariation between the rate of injection of histamine and its concentration in plasma is independent of the physiological action on the gastric mucosa. (The relations between this action and the histamine concentration will be discussed in detail in chapter 6.)

The results would permit the use of eq. (12) in the following discussions. If eq. (13) were valid it would be even more useful, but this has not been established. The present results and those of EMME-LIN & KAHLSON (1944), however, make this relation seem most probable, i. e. *the concentration of active histamine in the tissue is directly proportional to the injection rate of histamine.*

General reactions of the animals after histamine administration.

The administration of large doses of histamine (more than 3 mg/kg/hour) may cause a severe shock — DALE & LAIDLAW (1919) — and a considerable decrease of the blood pressure — MCCARREL & DRINKER (1941). In the present

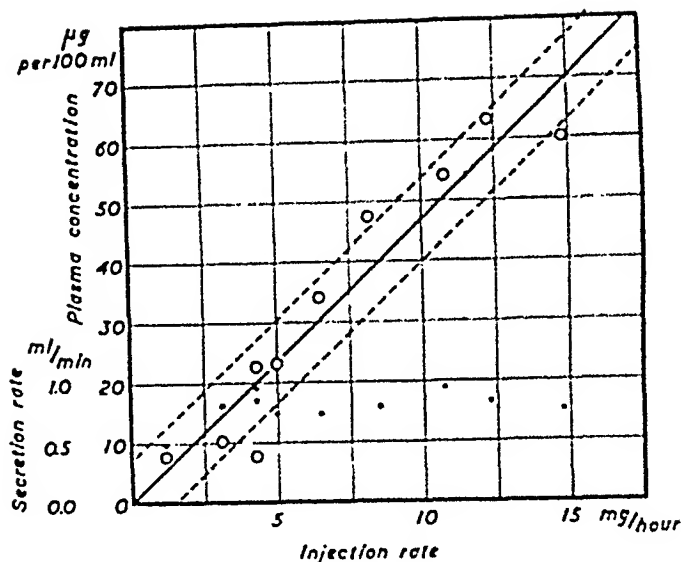


Fig. 11. The relation between the injection rate of histamine and the steady state values of the histamine concentration in plasma. The filled circles are the corresponding secretion rates. (The regression coefficient = 4.68; the standard error of estimate = ± 7.7 ; the coefficient of correlation = 0.94.)

experiments the largest dose has been about 0.6 mg/kg/hour and mostly not more than 0.25 mg/kg/hour were used. It is thought that these doses do not cause any persistent decrease in the blood pressure. It was only with the very highest injection rates that a slight dyspnoea was observed, but otherwise no undesired reaction could be noticed.

In a few experiments with narcotized cats (chloralose-urethane) the results of GIRAUD-COSTA & GAYRAL (1940) and EMMELIN (1945) have been confirmed, namely that the injection of small doses of histamine may cause an immediate decrease in the blood pressure which returns to its original level in a few minutes.

In what form does histamine exist in the plasma?

This question has been widely discussed by several authors. TARRAS-WAHLBERG (1936) considered that histamine occurred in the free form under special conditions. KAISER (1939) was of the opinion that histamine was bound to protein and inactive, which was also stated by ROCHA E SILVA (1942, 1943, 1944). EMMELIN (1945), from several kinds of experiments, concluded that histamine exists in plasma in a physiologically active form, or as an inactive compound from which histamine is extremely easily liberated. His results were criticized by COLLDLAHL, HOLMBERG & LAURELL (1946).

In the present experiments the dogs showed a high concentration in the plasma of substances which acted upon the guinea pig gut, even when no gastric secretion could be obtained. This activity may have been due to histamine, but there was no stimulation of the gastric glands. The reason might be a) that histamine occurred in a physiologically inactive form or b) that the gastric glands were in some way adapted to the actual histamine concentration and started the secretion only for an increase in the plasma histamine level.

In order to determine whether histamine alone was responsible for the gut contractions, the following experiments were performed:

Some *antihistamine* preparations were added to the Tyrode's solution in the intestinal bath. NILZÉN (1947) gives some concentrations of such drugs which he found necessary to inhibit the contractions. By using the same concentrations of *Antasten*¹ (Ciba) as Nilzén — 1.0 µg/ml — a pure histamine solution was rendered ineffective and the plasma extract was deprived of about 2/3 of its activity.

After a very large dose of *Lergitin*² (Recip) — 1 mg/ml — no contraction could be initiated either by pure histamine or by the plasma extract (the intestine did, however, still react on addition of acetylcholine).

These findings make it seem probable (but do not prove) that the "base values" of the plasma extracts were histamine.

It seems very difficult to disprove the cross-circulation experiments performed by EMMELIN (1945), which proved that histamine existed in an active form. On the other hand COLDAHL, HOLMBERG & LAURELL (1946) have disproved the existence of any adaptation. In the present experiments the "base value" was not constant and would sometimes show a marked decrease from day to day. This may also be in opposition to an adaptation theory.

It seems, on these findings, *probable* that histamine may occur in an inactive form in the blood.

CHAPTER 5.

Time-secretion Relation after Continuous Intravenous Injection of Histamine.

Effect of constant injection rates.

From chapter 4 the relation between the injection rate of histamine and the blood concentration is known. It is found that, in accordance with the theoretical considerations, *the blood concentrations will approach a steady state value* which is proportional to the injection rate. When the concentration in the blood is constant that in the tissues must also be considered to be constant as a constant blood concentration indicates a dynamic equilibrium between blood and tissue.

Considering the tissue concentration of histamine as following the theoretical curve in fig. 10, it will be of interest to know how the histamine effect in the gastric mucosa will proceed. *Fig. 12* shows a typical experiment, which gives an answer to the question. The injection of

¹ 2-phenylbenzylaminomethylimidazolin hydrochloride.

² Dimethylbenzylphenylethylendiamin hydrochloride.

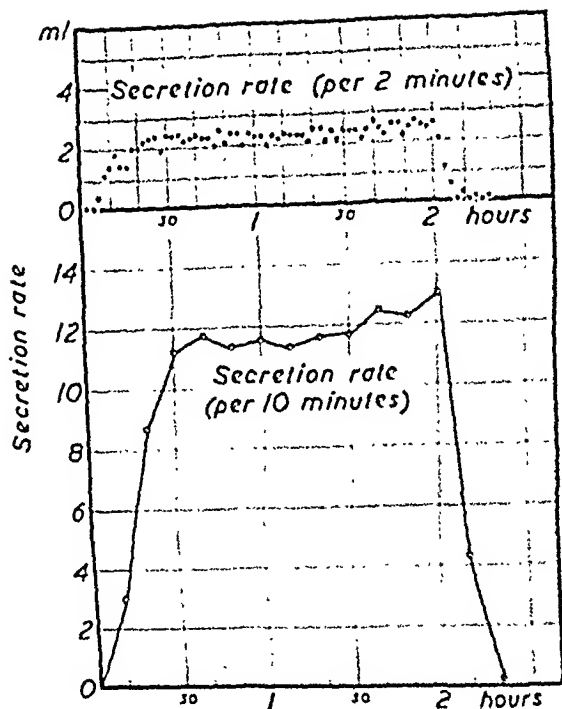


Fig. 12. A typical experiment showing gastric secretion after continuous intravenous injection of histamine. The injection is started at 0 hours and continued at a constant rate till 2⁰⁰ hours when it is stopped. The upper-part of the diagram shows the secretion rate expressed in ml/2 min; the lower part the corresponding ml/10 min.

histamine is started at time 0 and continued at a constant rate. After about 6 minutes the secretion starts and rises to a constant value which is reached in about 30 minutes. This means that there is a time lag of about 30 minutes before the rate of secretion reaches its final value, a fact which may be due to there being a similar time lag before the final steady value of the histamine concentration in the tissues is reached.

The secretion rates are expressed in ml/10 minutes (the lower part of the figure) and ml/2 minutes (the upper part). Cf. the "Procedure" chapter (page 14).

Several experiments have shown that a constant value of the secretion rate can be maintained for at least 8 hours. A sign of fatigue has been noticed only on one dog at high injection rates.

The general appearance of the curve resembles that of the time-concentration curve of a drug illustrating the theoretical considerations in chapter 4 (eq. 11 and fig. 10). Notice the time lag, which makes the curve concave upwards from the start at its origin. It is

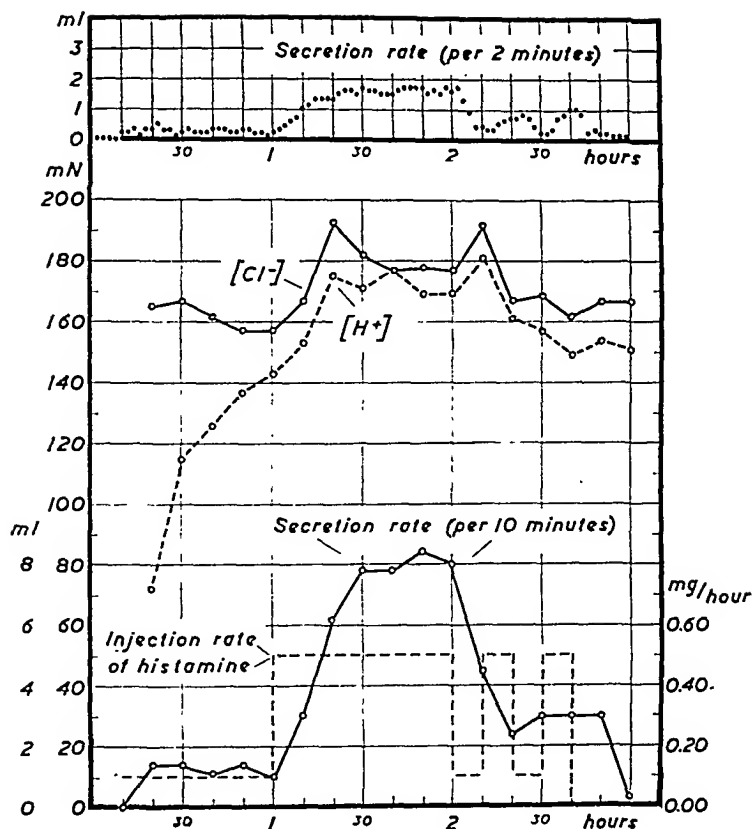


Fig. 13. The apparent disagreement between the injection rate of histamine and the secretion rate. This disagreement only appears when the amounts secreted over periods of 10 minutes are considered and is not apparent on consideration of the amounts secreted over periods of 2 minutes. (See text.)

not unexpected to find such a time relation, as the effect of histamine in inducing secretion must be related to its concentration in the tissue (mucosa). Cf. TEORELL (1937, c).

Effect of changing the injection rate of histamine.

The above mentioned results have the following interesting consequences. As the injected histamine has to pass through the blood stream before it enters the gastric mucosa, a 30 minutes time lag will occur between a change in the injection rate and a new steady state value of the gastric secretory rate.

This may sometimes cause surprising results, which have been seen in experiments of which a typical one is given in fig. 13. When a sufficient time has elapsed after starting the injection, and the secretory rate (s. r.) has become constant, the injection rate (i. r.) is sud-

CHAPTER 6.

Relation between Histamine Concentration and Rate of Secretion. Concentration-action Curves.

Theoretical considerations.

Histamine acts directly upon the peripheral structure of the gastric mucosa. It has been proved many times, that no nerve supply by the vagus to the stomach is required for its response to histamine — POPIELSKI (1920), KEETON, LUCKHARDT & KOCH (1920), KOSKOWSKI (1922) and IVY & JAVOIS (1924). Even a transplanted piece of mucosa secretes hydrochloric acid after stimulation with histamine — IVY & FARRELL (1925), KLEIN & ARNHEIM (1932) and GROSSMAN & IVY (1946). *It may therefore be expected, that a close relation will be found between the histamine content of the mucosa and its secretion rate.* — cf. TEORELL (1937 c).

LIPS, VERSCHURE & STRENGERS (1947) could not find any covariation between histamine concentration in the blood and gastric acidity, but this may be due to the specific experimental procedure employed.

The mechanism in which histamine takes an active part is unknown. We can, however, make a rough model of the system which may incorporate the mechanism while remaining ignorant of its details.

Define the secretory process as the “*producing flow*,” which is converted into hydrochloric acid by means of histamine.¹ Consider the maximal response of the mucosa towards histamine to be a ml/min., which will be secreted when all of the “*producing flow*” is converted into hydrochloric acid.

At a certain concentration of histamine in the mucosa, z ,² some part — or the whole of it — is causing secretion of gastric acid.³

We may call the *rate* of this contribution of histamine or better flux of histamine to the secretory process ϕz . This ϕz is certainly proportional to z and consequently to the injection rate of histamine r . (Cf. eq. (13).)

¹ This “*producing flow*” may be the production of a real precursor to the HCl or of different chemical compounds which take part in the acid formation.

² Cf. eq. (13).

³ Histamine may be consumed during the secretory process or act in a catalytic way. The following argument will be the same in both cases.

Thus we may put

$$\phi z = k_1 r \quad (17)$$

ϕz is converting the "producing flow" into hydrochloric acid at a rate of v ml/min.

If ϕz is increased by $d\phi z$ this increment may react with the "producing flow" still left, $(a - v)$, and thus cause an acceleration of the secretion, dv . Following the law of mass action dv will be proportional to the product of $d\phi z$ and $(a - v)$ or

$$dv = K(a - v) d\phi z \quad (18)$$

where K is a constant.

Integration of this equation gives

$$v = a(1 - e^{-K\phi z}) \quad (19)$$

or by substitution of (17) and putting $Kk_1 = k$

$$v = a(1 - e^{-kr}) \quad (20)$$

This is a well known mathematical expression. It is graphically represented by a curve passing through the origin and rising exponentially to an asymptotic value a . — cf. ÖBRINK (1947 b).

Experimental data.

In order to get satisfactory experimental results in this field, the adoption of the method of continuous intravenous injection affords a great help in getting constant and easily determined secretory rates. In the present experiments the constancy has been observed for at least fifteen 2-minute periods and the mean of the secretory rate has been calculated. The values are expressed in ml/min (not in ml/2 minutes).

From the experiments on one dog (19.7 kg) the injection rates of histamine and the corresponding rates of secretion are given in Table 1. The third column in this table shows that the injection rates have been changed at random. The concentration of histamine injected has also been varied as is seen in the second column.

Plotting the secretion rates as the ordinate in a coordinate system with the rates of injection of histamine as the abscissa we get the fig. 14.

How does eq. (20) fit these points? That is a matter of correlation analysis.

Table 1. *The relation between injection rate of histamine and secretion rate of gastric juice.*

| Date | Conc. of histamine injected mg/ml | Rate of injection mg/hour | Rate of secretion ml/min. |
|------|-----------------------------------|---------------------------|---------------------------|
| 31/1 | 0.50 | 3.40 | 1.39 |
| 5/2 | 0.75 | 2.85 | 1.06 |
| 9/2 | 0.75 | 2.85 | 0.96 |
| 11/2 | 0.25 | 0.95 | 0.54 |
| 11/2 | 0.25 | 4.80 | 1.18 |
| 12/2 | 0.10 | 0.40 | 0.21 |
| 12/2 | 0.10 | 1.90 | 0.98 |
| 13/2 | 0.10 | 0.40 | 0.30 |
| 13/2 | 0.10 | 0.68 | 0.41 |
| 14/2 | 0.25 | 0.95 | 0.43 |
| 14/2 | 0.25 | 1.30 | 0.67 |
| 15/2 | 1.00 | 3.80 | 1.03 |
| 15/2 | 1.00 | 5.20 | 1.25 |
| 20/2 | 0.20 | 0.54 | 0.35 |
| 20/2 | 0.20 | 1.50 | 0.83 |
| 20/2 | 0.20 | 3.92 | 1.17 |
| 25/2 | 0.30 | 1.03 | 0.47 |
| 25/2 | 0.30 | 2.34 | 0.89 |
| 26/2 | 0.30 | 1.03 | 0.52 |
| 28/2 | 0.50 | 1.78 | 0.91 |
| 28/2 | 0.50 | 3.50 | 1.34 |
| 1/3 | 0.33 | 1.29 | 0.52 |
| 1/3 | 0.33 | 2.06 | 0.88 |
| 1/3 | 0.33 | 2.53 | 1.08 |
| 4/3 | 0.03 | 0.10 | 0.02 |
| 4/3 | 0.03 | 0.14 | 0.03 |
| 4/3 | 0.03 | 0.18 | 0.11 |
| 8/3 | 1.00 | 2.70 | 0.98 |
| 8/3 | 1.00 | 4.50 | 1.13 |
| 20/3 | 1.00 | 2.50 | 1.04 |
| 20/3 | 1.00 | 5.40 | 1.15 |
| 20/3 | 1.00 | 6.50 | 1.20 |

Mathematical treatment of the experimental data.

The method of least squares has been employed.¹

¹ For technique see M. EZEKIEL: *Methods of Correlation Analysis*. New York 1945.

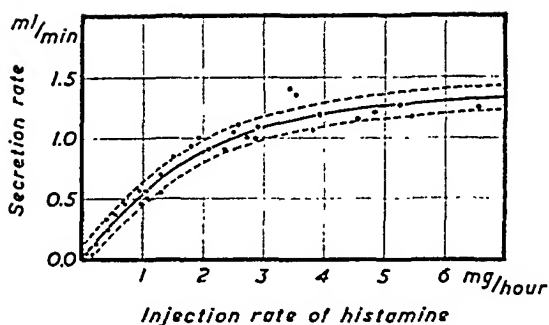


Fig. 14. The relation between the injection rate of histamine (r) and secretion rate of gastric juice (v). The smoothing curve is that obtained from the eq. $v = 1.37 - 1.38 e^{-0.5r}$. The dotted lines indicate the "standard error of estimate."

The eq.

$$v = a(1 - e^{-kr})$$

may be written in a more general form

$$v = a_0 - a_1 e^{-kr} \quad (21)$$

where the condition that the curve must pass through the origin is abandoned.

Eq. (21) may be written

$$v = a_0 - a_1 u \quad (22)$$

where

$$u = e^{-kr} \quad (23)$$

Eq. (22) gives the following normal equations:

$$\begin{cases} n \cdot a_0 - a_1 \sum u = \sum v & (24) \end{cases}$$

$$\begin{cases} a_0 \sum u - a_1 \sum u^2 = \sum uv & (25) \end{cases}$$

The values of u are calculated from (23) and will depend on the value of k . It is therefore necessary to solve the eq. system (24), (25) for different values of k and calculate the corresponding sum of squares of the differences, d , which will have the following expression:

$$\sum d^2 = \sum v^2 - a_0 \sum v + a_1 \sum uv \quad (26)$$

In Table 2 the dependence of this sum on the value of k indicates that the least sum is obtained for a k value of about 0.5. A more exact determination of the value would have no mathematical significance.

Table 2. The statistical value of eq. (21) for different k -values.

| k -value | Σd^2 | a_0 | a_1 |
|------------|--------------|-------|-------|
| 0.25 | 0.5670 | 1.79 | 1.64 |
| 0.50 | 0.2823 | 1.37 | 1.38 |
| 0.75 | 0.2866 | 1.21 | 1.35 |
| 1.00 | 0.4093 | 1.13 | 1.36 |

Thus the solution is

$$k = 0.5$$

$$a_0 = 1.37 \pm 0.03$$

and
$$a_1 = 1.38 \pm 0.06$$

Thus

$$v = 1.37 - 1.38 \cdot e^{-0.5r} \quad (27)$$

The index of correlation, R , is 0.97 ± 0.01 .

The standard error of estimate, $\sqrt{\frac{\Sigma d^2}{n-2}}$ is 0.09 ml/min. The eq. (27) gives a very good agreement as is seen in *fig. 14*, where the broken lines are the statistical limits given by \pm the standard error of estimate.¹ It is seen from this figure and understood from the a -values, that at an injection rate $r = 0$ the curve has a v -intercept, that is not statistically different from 0, i. e. *the curve passes through its origin*.

Thus it would be correct to write the eq. (27)

$$v = 1.37 (1 - e^{-0.5r}). \quad (28)$$

The experiments above were performed on a single dog. The same results, however, are obtained on other dogs. For example *fig. 15* shows the same relations on this and other dogs and indicates, that *it might be justifiable to consider the above results as having general validity*.

¹ Other mathematical expressions have been correlated with the experimental values but with unsatisfactory results. Examples of such equations are:

$$y = a e^{-\frac{1}{x}} + b;$$

$$y = a \cdot \log (x + b);$$

$$ax^2 + bxy + cy^2 + dx + ey + f = 0;$$

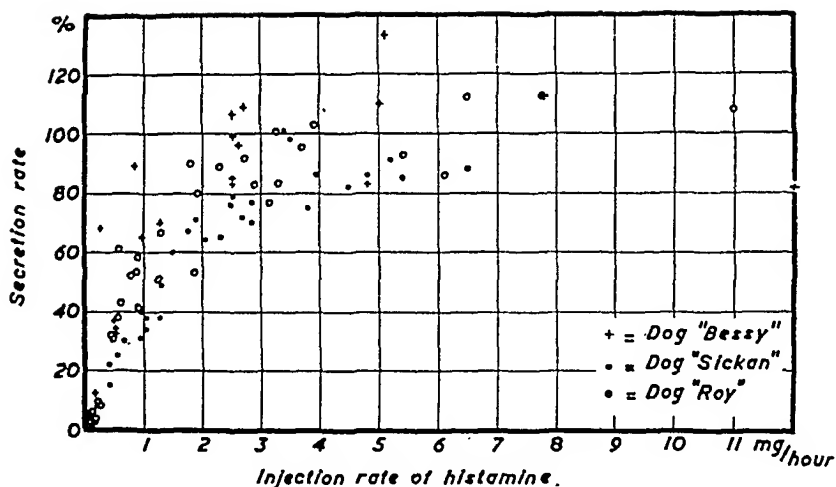


Fig. 15. A comparison between concentration-action curves from different dogs.

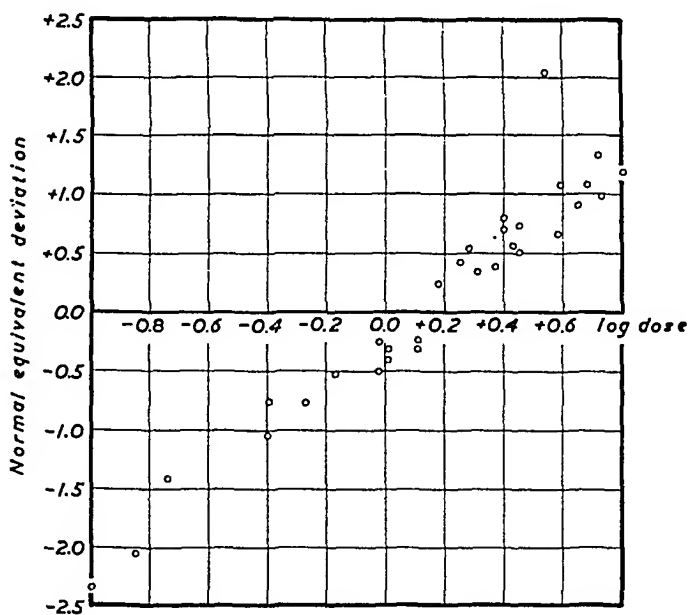


Fig. 16. The probit transformation of the experimental results in fig. 14.

These results suggest that the secretory rate is very closely related to the injection rate of histamine and to its concentration in the plasma and probably also to the concentration in the mucosa. This is in perfect accordance with the results presented in the previous chapter, where lack of agreement between the stimulus and secretion was only apparent and was due to special experimental circumstances.

Discussion on the concentration-action curve.

Fig. 14 shows the close relation between the injection rate of histamine (i. e. the concentration of histamine) and the secretion rate. This shape of a concentration-action curve is well-known to all physiologists and pharmacologists.

In the discussion of this curve it might be of interest to transform the abscissa (the *dose*) into a logarithmic scale (the *log dose*). With this transformation fig. 14 changes to a slightly *S*-shaped curve. This relation was also reported by IVY, HANSON & GROSSMAN (1947).

The *S*-curve may be considered as the integral of a normal distribution curve as indicated by the fig. 16 where the "normal equivalent deviation" to the secretion rate¹ is plotted against the log injection rate. This follows a procedure proposed by GADDUM (1933) and would give a straight line if the increments of the secretion rate in relation to the logarithm of the injection rate of histamine resulted in a normal distribution curve. The points in fig. 16 lie practically along a straight line.

This means, in practice, that the procedure presented is a good method for standardizing histamine preparations. The intersection between the straight line and the abscissa in fig. 16 is the corresponding "*LD 50*" — TREVAN (1927) — and this dose is a good measure of the activity of the histamine preparation. (Because of the laboriousness of this method it is, however, only of theoretical interest.)

It is of interest to compare the present concentration-action curve with the one obtained when the *pancreas* is stimulated by the continuous intravenous injection of *secretin*. WILANDER & ÅGREN (1932) and GREENGARD, STEIN & IVY (1941) found an *S*-shaped relation between the secretion rate and the *dose* of *secretin*. Their technique is similar to the present one, but the resulting curves are different.

It is not possible to explain this disagreement on the basis of our present knowledge.

¹ "The normal deviation (*y*) which is equivalent to any given percentage depends on the shape of the normal curve. It is equal to the deviation from the mean, and is measured in standard deviations as units. When the percentage (*p*) is 50, *y* = 0; when it is greater than 50, *y* is positive, and when it is less than 50, *y* is negative. Algebraically its value is given by the equation

$$p = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^y e^{-\frac{y^2}{2}} dy$$

(After GADDUM (1933).)

As the volume secreted is produced by the activity of many cells, the mode of reaction of these elements, ought to be known before we can disintegrate the resultant action curve.

At the moment we may only give an outline of the possible modes of reaction of the secreting cells:

a) The cells may react uniformly, i. e. they start the secretion at the same dose and have a *graded response* to increasing doses. The concentration-action curve of every individual cell would then be expected to have the same shape as the curve in fig. 14.

b) The cells may have a *quantal*, or all-or-nothing, response, i. e. they start reacting at a certain threshold dose and at once give a maximal response. If the threshold doses were to have a distribution curve skew to the right fig. 14 might be the result.

c) Finally the cells may have a graded response but start secretion at different threshold doses.

It is impossible to say which of these three possibilities is the correct one.

CHAPTER 7.

The Secretion of Gastric Juice after Subcutaneous Injections of Histamine.

As the most usual way of stimulating the gastric glands by histamine is by subcutaneous injection, it seems necessary to compare this route of administration with the continuous intravenous one from a kinetical point of view.

From chapter 4 the time-concentration relation in blood and tissue after a subcutaneous injection was assumed to be known (eqs 8, 9 and fig. 9, page 21). Combining eq. 9 (the tissue concentration) with the relation "tissue concentration"—"secretion rate" (eq. 20, fig. 14, page 33) the kinetics of the secretion rate after a subcutaneous injection of histamine will appear. The mathematical expression of this relationship is, however, rather complicated¹ but the general shape of the curve is much the same as the tissue concentration curve in fig. 9 (page 21).

¹ $v = a [1 - e^{-k(R_1 e^{m_1 t} + R_2 e^{m_2 t} + H e^{-k_1 t})}]$. The meanings of the symbols are the same here as in chapters 4 and 6.

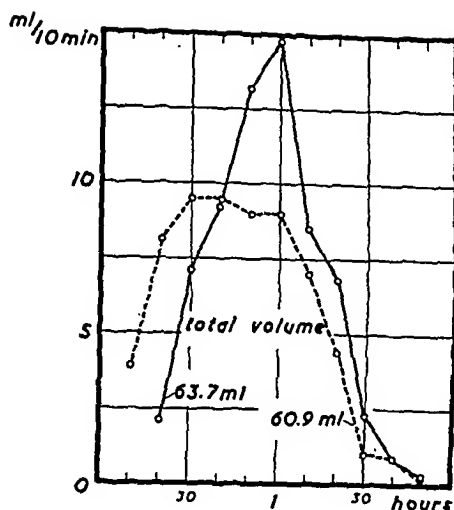


Fig. 17. Two results from subcutaneous injections of histamine. (4.0 mg.) The resorption conditions were different, which resulted in different maximal secretion rates. The total amounts secreted were, however, almost equal.

This course is quite well known to everyone who has followed the gastric secretion after a subcutaneous injection of histamine. After a time lag of about 6—10 minutes the secretion rate increases to a maximal value which, in pouch dogs, is obtained after about 25 minutes, when 0.5—1.0 ml of histamine has been injected (the dose is insignificant). In about 60 minutes the period of secretion is over.

The secretion after a single subcutaneous injection of histamine.

Usually, and especially in clinical practice, a single subcutaneous injection of histamine is used. Either the maximal rate of secretion or the total amount secreted is observed.

The maximal rate of secretion.

In chapter 4 it was suggested that the maximal value of the histamine concentration in the tissue was dependent on the resorption rate from the depot (page 23). If this can be kept constant from injection to injection (i. e. k_1 is constant) a close relation between the dose and the maximal rate of secretion would appear. In order to determine such a maximal rate of the secretion it was, however, stated (page 29) that it is necessary to collect at short intervals. Even 10-minutes' intervals may be too long.

If furthermore the resorption varies, the maximal values may differ considerably. In *fig. 17* the same dose (4.0 mg histamine dihydro-

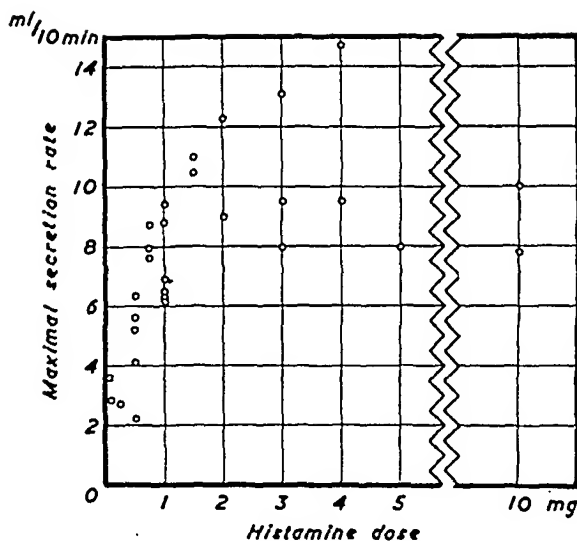


Fig. 18. The relation between the dose of histamine given subcutaneously and the maximal values of the secretion rate.

chloride dissolved in 2 ml saline) was given on two different days to the same dog. In the one experiment the injection was made on the thigh where a distinct swelling occurred, in the other one in the inguinal region where the whole volume was spread out. In the second case the maximal secretion was much higher than in the first one, which may depend to some extent on the different conditions of resorption from the two depots. Theoretically we would expect the second curve (the high one) to reach its maximum before the first one (the flat one) but for some unknown reason this was not the case.

If we try, however, to make the resorption conditions as nearly identical as possible, the relation between the dose of histamine and the maximal secretion value (per 10 minutes) will be as good as in *fig. 18*. For small doses this relation seems satisfactory.

The total volume secreted.

The total amount of the drug in the tissue was found to be independent of the resorption rate (independent of k_1) as shown in eq. (16) (page 22): $Z = \frac{k_2}{q} N_0$, where Z = the total amount of the drug present in the tissue after a subcutaneous injection of a dose N_0 ; k_2 and q are constants.

As, however, the relation between the concentration of histamine in the tissue and the secretion rate is not a direct proportionality

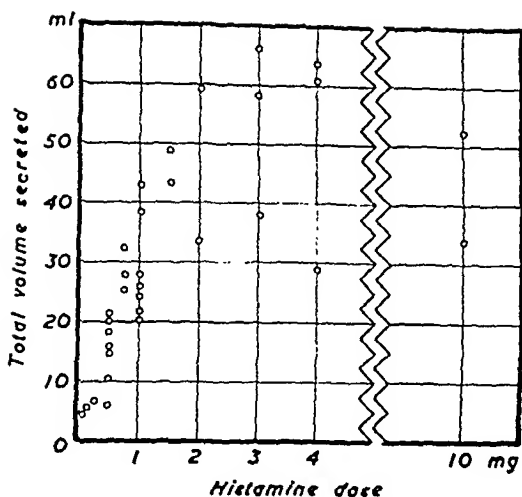


Fig. 19. The relation between the dose of histamine given subcutaneously and the total volume gastric juice.

except at the lowest concentrations (eq. 20, page 31 is almost a straight line near to the origin), we might expect to find a close relation between the dose of histamine and the total amount secreted only when the tissue concentrations are kept at a moderate level.

If this is the case the total amount secreted will be proportional to the dose of histamine even though the maximal secretion values per 10 min. period differ. Though the experiments in fig. 17 were performed with rather large doses of histamine (4 mg) they nevertheless show a good agreement in respect to the total volumes (60.9 and 63.7 ml).

It can, however, be concluded that, owing to the shape of the relation "tissue concentration"—"secretion rate," a good agreement between the subcutaneous dose of histamine and the total amount secreted will only be obtained for small doses, which is shown to be the case in fig. 19.

"Continuous histamine test."

One interesting consequence arising in this connection is the behaviour of the tissue concentration after *frequently repeated subcutaneous injections*. Such a method of administration was adopted by BUCHER, IVY & GRAY (1941) and others, under the name of "*continuous histamine test*". Small doses of histamine were injected subcutaneously every 10 minutes with the intention of obtaining a constant secretion rate. In this case the concentration of histamine in the tissue may be considered as the sum of the concentrations obtained after every individual injection. This is best understood by looking at fig. 20. The time lag between an injection of histamine and its maximal concentration in the tissue

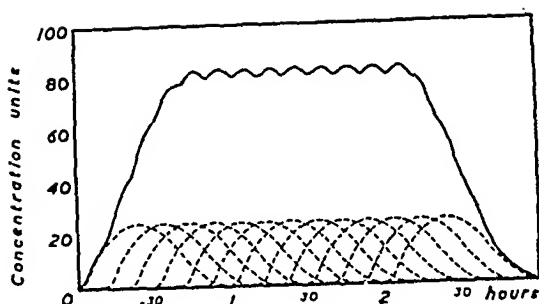


Fig. 20. The theoretical concentrations of histamine in the tissue after repeated subcutaneous injections. Every single injection is assumed to give a time-concentration curve like the dotted ones. The sum of these gives the total concentration.

is assumed to be 25 minutes. After about 50—60 minutes the tissue concentration level will oscillate around a mean value. The secretion of gastric juice is supposed to follow these oscillations and will consequently give the same secretion volume for every period of 10 minutes as long as the injections are repeated. This method might therefore be almost as good as the one used in this paper. A disadvantage is the repeated injection injuries.

The method presumes, however, that the resorption rate (the “velocity constant” k_1 , page 20) from the injection depot is constant, otherwise the individual dotted curves in fig. 20 will not be identical and a constant secretion rate will not be obtained.

CHAPTER 8.

The Minimal Effective Dose of Histamine Producing Gastric Acid.

It is apparent that histamine acts after its transportation by the blood—GUTOWSKI (1924 b)—to the cells of the mucosa. In a previous chapter the dependence of the secretion rate on the concentration of histamine in blood and mucosa has been demonstrated.

In this connection it seems convenient to discuss a problem which has been a point of controversy for many years, i. e. the minimal effective dose of histamine in the production of gastric acid.

The authors who have given the question any attention have never doubted the existence of a threshold for histamine action on the gastric mucosa but they do not agree as to its level. Table 3 shows the minimal effective doses as postulated by different authors.

Study of the curve in fig. 14 (page 33) allows a contribution to the problem to be made.

Table 3. *The minimal effective dose of histamine as reported by different authors.*

| Author | Reported threshold dose | Expressed in $\mu\text{g}^1/\text{kg}/\text{hour}$ |
|---------------------------------------|--|--|
| IVY and JAVOIS (1924) | 0.0027 $\text{mg}^1/\text{kg}/\text{min.}$ | 162 |
| TEORELL (1933)..... | 100 $\gamma^2/15 \text{ min.}^4$ | 58 |
| ENGESTRÖM (1935) | $< 5\gamma^3/\text{kg}/15 \text{ min.}$ | < 12 |
| McELIN and HORTON (1946) | $< 0.00003 \text{ mg}^1/\text{kg}/\text{min.}$ | < 1.8 |
| IVY, HANSON and GROSSMAN (1947). | 0.03 $\mu\text{g}^1/\text{kg}/\text{min.}$ | 1.8 |
| The present author (Dog S) | | 3 |
| » » » (Dog R) | | 0.9 |
| » » » (Dog B) | | 0.8 |

¹ Histamine base.

² Histamine phosphate.

³ Histamine dihydrochloride.

⁴ On a cat (probably about 2.5 kg in weight).

(Histamine base : histamine phosphate : histamine dihydrochloride = 1 : 2.79 : 1.67.)

If a threshold value exists it will be at the point where this curve cuts the abscissa, i. e. the r value for $v = 0$. The eq. (27) indicates the r value to be 0.01 mg/hour , but the statistical limits of the curve enclose the origin and thus the threshold value is not different from 0. That means, that *the slightest amount of histamine administered by continuous intravenous injection will initiate secretion.*

The least injection rate used in the experiments on the dog shown in fig. 14 was 0.1 mg/hour or 0.005 $\text{mg}/\text{kg}/\text{hour}$. In another dog injection rates as low as 0.0013 $\text{mg}/\text{kg}/\text{hour}$ / i. e. 0.0008 mg histamine base/ kg/hour were effective. This is the lowest value given in the literature as far as is known to the author (cf. Table 3).

Therefore it seems justifiable to state, that *there is no threshold value for the effect of histamine on gastric secretion.*

It is, however, necessary to point out, that this conclusion is valid only for histamine *administered* to the body. It ought to be kept in mind, that *there exists histamine in the blood of a non-treated dog, which has no secretory effect* as described in chapter 4 (page 24). This histamine is not taken into account above.

CHAPTER 9.

On the Primary Acidity.

It is well known that the acid produced by the gastric mucosa will be exposed to dilution, neutralization, diffusion and other possible processes, which may alter its original strength. The concentration of the gastric acid as measured in a sample obtained from a whole stomach or a pouch will therefore have a different value than it would have if it were examined at the very moment of secretion. This acid in *statu nascendi* has a concentration, which is usually called the *primary acidity*, (*Pa*). In an analogous way the concentration of the changed acid may be named the *secondary acidity*, (*Sa*).

Ever since HEIDENHAIN and PAVLOV first suggested the existence of a primary acidity two fundamental questions have been raised:

- 1) what *value* has the *Pa* and
- 2) is it *constant* or not, i. e. is it independent of the rate of secretion?

In spite of the many attempts of several authors to answer them the questions are still open.

According to the second question PAVLOV (1898 p. 38—39) and his school thought that the *Pa* must be constant, a suggestion that he never tried to prove experimentally but in which he had very good reason to believe (cf. chapter 10 page 46). Also cf. HOLLANDER & COWGILL (1931), HOLLANDER (1932, 1934, 1938).

ROSEMAN (1907) was the best known of the opponents of this view. He noticed that in spite of a variability of the *Sa* the chloride content remained constant, and he considered the secretory process to be an elaboration of a neutral chloride which by some specific stimulation was converted into HCl. The stronger the stimulation the more HCl was formed and the less neutral chloride was left.

His conclusions were also adopted by BERGLUND, JOHNSON & CHANG (1935) who considered the secretion of the chlorides as the essential part of the parietal secretion.

Experiments performed by the present author, however, suggest (page 71 ff.) that the entrance of chlorides in the gastric juice is influenced not only by the acid secretion but also by diffusion processes.

Other theories about the *Pa* have also been proposed, but they are only variants of the two mentioned, cf. ENGSTRÖM (1935).

Those workers, who, like Pavlov, believe in a constant strength of the Pa have been interested to know the *value* of it.

According to PAVLOV the decrease of the Pa was due to *dilution and neutralization by mucus*, the effect of which would have a greater significance at low secretion rates. The higher the secretion rate the less the difference between Pa and Sa. Therefore HOLLANDER (1934) tried to evaluate the magnitude of the Pa by *extrapolation* of the Sa values for infinitely large secretion rates. He found that the Pa should be about 170 mN — a most probable value because it is almost *isotonic with blood*. Using the same method GRAY (1943) gives 159 mN as the Pa value.

In a few cases experiments have been made to determine the Pa by a dilution method. The principles were to cause such a high dilution of the primary acid, that the reduction of acidity by dilution with mucus or other admixtures of the gastric juice would be negligible. These observations gave similar results to the extrapolation method, though in a few cases extremely high values were obtained — WILHELMJ, O'BRIEN & HILL (1936), PENNER, HOLLANDER & POST (1940), BANDES, HOLLANDER & GLICKSTEIN (1940).

When TEORELL (1933) proposed his theory of diffusion (the "*diffusion theory*" cf. page 47) another factor, that may reduce the Pa had to be taken into consideration. Using this new principle TEORELL (1940) described another method of determining the Pa. He caught the secreted acid in a *glycocol buffer*, thus causing both a dilution and a prevention of back diffusion of the hydrogen ions. Using this method he concluded that the Pa was constant and almost isotonic with blood (see also the papers by IHRE (1938), ELLIOT, RISHOLM & ÖBRINK (1942) and KAIJSER (1943)), but he also observed much higher values, especially at low secretory rates. These values were, however, too uncertain and not taken into account.

By the method described in chapter 2 it has, however, been possible to make observations at very small secretion rates. Under these new conditions work on the Pa has been carried out by LINDE, TEORELL & ÖBRINK (1947). Their results are of importance for the discussion of the Sa, that follows, and will therefore be recapitulated here.

The primary acidity is probably not constant but becomes higher at low secretory rates. It decreases as the secretion rate increases and approaches a value of about 170 mN asymptotically. *Fig. 21* shows

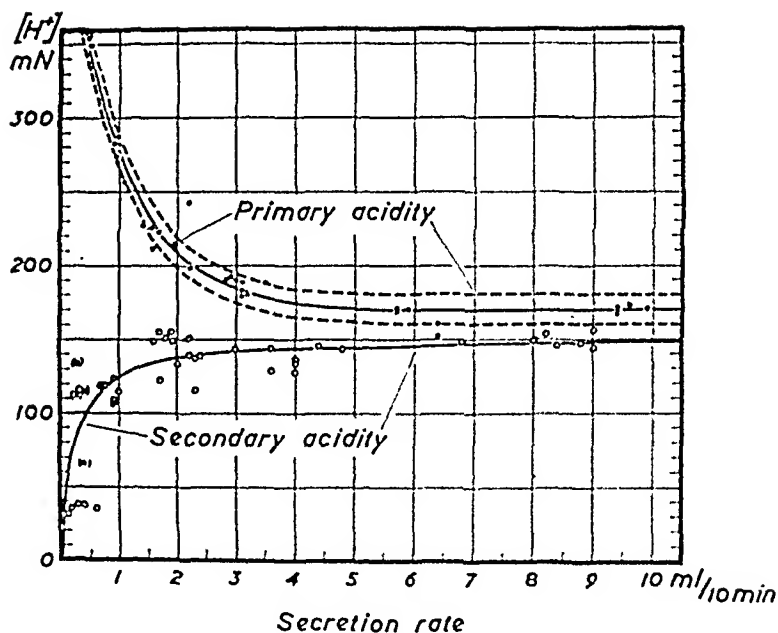


Fig. 21. The primary acidity in relation to the secretory rate. The corresponding secondary acidity values from the same dog are plotted as open circles. — After LINDE, TEORELL & ÖBRINK (1947).

the relation. In this figure the relation between the secretion rate v and P_a is expressed in

$$P_a = P_\infty + (P_0 - P_\infty)e^{-k_1 v} \quad (29)$$

where P_∞ is the P_a -value for $v = \infty$, P_0 the value for $v = 0$ and k_1 is a constant. The corresponding S_a -values are given for comparison.

The corresponding chloride values are always somewhat higher which may depend on the diffusion of Cl into the buffer solution.

The significance of the values has been critically discussed with regard to the possibilities of artefacts.

One should note the good agreement between these high P_a -values and some modern theories about the HCl -formation in the mucosa. BULL & GRAY (1945) consider the exchange diffusion of the anion from an organic acid for Cl^- to be the essential process in the HCl -formation. The organic acid is formed from glucose and may be pyruvic acid, which must have a concentration of 330 mN in order to be isotonic with blood. Conversion of this into HCl will of course give an HCl -solution of 330 mN which is hypertonic because of the high degree of ionization of hydrochloric acid. In their experiments LINDE, TEORELL & ÖBRINK obtained values of about 350 mN.

It should, however, be noticed that the osmotic work required to make such a hypertonic solution, may not have been performed in the buffer solution experiments, as the isotonic volume instilled probably prevented any significant hypertonicity even if all the pyruvic acid was converted to HCl.

As yet therefore, there are no *definite* proofs of the existence of these extremely high Pa values.

As will be seen in chapter 10 it is of no decisive importance for the following whether the Pa increases at low secretory rates or remains constant, and therefore the value of the Pa will mostly be handled as a constant in the following.

CHAPTER 10.

Regulation of the Acidity.

Introduction.

If we assume a *primary acidity* (Pa), the concentration actually observed, the *secondary acidity* (Sa), results from a reduction of the Pa. The difference between Pa and Sa must depend on some mechanism about the nature of which a discussion has been going on for many years.

PAVLOV (1898) who was the first to pay any attention to this question, stated that the *mucus* would *neutralize* and *dilute* the secreted acid. He noticed that the acidity decrease was more pronounced at low rates of secretion. The capacity of mucus for binding hydrochloric acid could be detected in a convincing way at the start of an experiment. At the height of the secretion, the mucus was, however, maximally loaded with acid and when the rate decreased at the end of an experiment no decrease in acidity would therefore be possible. This made Pavlov believe in a constant primary acidity (cf. page 43) and in the reduction of acidity by mucus. This theory concerning the mucus seemed very convincing and is still defended. BABKIN and his co-workers believe in the neutralizing capacity of mucus.¹

BOLDYREFF (1907, 1911, 1914, 1933) has proposed another explanation of the acidity decrease. He observed that under certain conditions

¹ For a good review in this field see the monograph by BABKIN (1944).

a *regurgitation of duodenal contents* occurred. This admixture was alkaline and able to neutralize (and of course dilute) the acid. The neutralizing capacity of the duodenal juice is considered to be much higher than that of the mucus. According to IHRE (1938) the content of alkali is about 10 mN in the mucus but 100 mN in the pancreatic juice.

In most cases this explanation of BOLDYREFF is very difficult to exclude. However, experiments have been carried out on man in which precautions have been taken against getting any admixture from the intestine. Cf. IHRE (1938) and ELLIOT, RISHOLM & ÖBRINK (1942).

In the present paper the experiments have been performed on pouch dogs. That means that no account need be taken of any admixture from places outside the pouch.

In 1933 TEORELL put forward his *theory of diffusion*. By experiments on cats' stomachs he came to the conclusion that the gastric mucosa could be considered as similar to a dialysing membrane, through which the hydrogen ions of the juice were exchanged for sodium ions of the blood (or cells) — cf. also TEORELL (1935 a, 1939, 1940, 1947).

His statement was based on the fact that a considerable decrease in the concentration of instilled hydrochloric acid occurred in a tied-off stomach without any appreciable changes in the volume.

Some papers have appeared — IHRE (1938), ELLIOT, RISHOLM & ÖBRINK (1942), KAIJSER (1943), FLENSBORG (1944), SHAY, KOMAROW, SIPLER & FELS (1946) — that give support to the theory of diffusion, but there are unexplained features, a fact which has been pointed out by some authors — see e. g. BABKIN, HEBB & KREUGER (1941), BABKIN (1944). Of great importance are the papers by COPE, COHN & BRENIZER (1943) showing that radioactive sodium is absorbed from a gastric pouch and by COPE, BLATT & BALL (1943) showing that heavy water easily penetrates the gastric mucosa.

This paper is an attempt to throw some light upon remaining problems in the acidity regulation. First, however, it is necessary to give a more detailed account of the theory of diffusion.

The "Diffusion Theory."

First it must be pointed out that diffusion in this case does not necessarily mean a penetration of the hydrogen ions *through* the

mucosal membrane, but that ad- or absorption *may* have the same effect in producing an acidity decrease under special conditions (cf. page 51).

Three ions are of particular interest and they will be considered here. These are the hydrogen, the chloride and the sodium ions. In this chapter only the behaviour of the hydrogen ion will be discussed. According to TEORELL (1947) the theoretical considerations are the following:

As pointed out by GUGGENHEIM it is allowable to consider the diffusion of the different *ions* of HCl and NaCl resp. taking place as undissociated molecules.

Thus the changes in the *concentration* of the hydrogen ions in the stomach are the following:

$$\text{increment due to secretion} = \frac{v C_0}{p + vt} dt \quad (30)$$

$$\text{decrement due to diffusion} = - \frac{k H}{p + vt} dt \quad (31)$$

$$\text{decrement due to dilution} = - \frac{v \cdot dt}{p + vt} \cdot H \quad (32)$$

where v is the secretion rate, p is the volume of non-acid material present in the stomach when the experiment starts (test meal volume), C_0 the strength of the secreted acid (the primary acidity at the secretion rate v), H the actual hydrogen ion concentration, t the time and k a permeability coefficient including the diffusion coefficient, the diffusion area and the thickness (resistance) of the membrane. The total changes of the hydrogen ion concentration will be

$$\frac{dH}{dt} = \frac{v C_0 - (k + v) H}{p + vt} \quad (33)$$

The solution of this differential eq. will be

$$H = \frac{C_0}{\frac{k}{v} + 1} \left[1 - \frac{1}{\left(1 + \frac{vt}{p} \right)^{1 + \frac{k}{v}}} \right] \quad (34)$$

If no test meal volumes are present

$$H_{p=0} = \frac{C_0}{\frac{k}{v} + 1} \quad (35)$$

This means that the secondary acidity is not dependant on the time, but only on the secretory rate.

By putting $v = 0$ in eq. (35), $H = 0$, and when $v = \infty$, $H = C_0$.

That means, that the diffusion (like the neutralization and dilution) is of greater significance at low secretory rates.

The deduction made above, which is to be found in the recent paper by TEORELL (1947) assumes the secreted volume to be instantly and thoroughly mixed with the volumes previously secreted. This makes it justifiable to put the "decrement due to diffusion" in eq. (31) as proportional to H .

If on the contrary no mixing occurs the diffusion gradient will always be $C_0 - 0^1$ (instead of $H - 0$)¹ as it will only be the freshly secreted volume that is in contact with the diffusion area.

This proposition makes the eq. (33) change to

$$\frac{dH}{dt} = \frac{(v - k) C_0 - vH}{p + vt} \quad (36)$$

which gives

$$H = C_0 \left(1 - \frac{k}{v}\right) \left[1 - \frac{1}{1 + \frac{vt}{p}}\right] \quad (37)$$

or if $p = 0$

$$H = C_0 \left(1 - \frac{k}{v}\right) \quad (38)$$

Here $H = 0$ when $v = k$ and $H = C_0$ when $v = \infty$.

Eq. (38) represents a hyperbolic curve approaching $H = C_0$ for increasing v values and cutting the v -axis at $v = k$ (cf. page 69).

A curve, almost identical with this one is given by GRAY, BUCHER & HARMAN (1941) but on a different theoretical basis (cf. page 69).

The value of the primary acidity (C_0).

An important question is the value of C_0 . In the previous chapter it was found that C_0 varied with the secretion rate in accordance with the eq. (29):

$$Pa = P_\infty + (P_0 - P_\infty) e^{-k_1 v}$$

where Pa is the primary acidity, P_∞ the Pa value for $v = \infty$, P_0 the Pa value for $v = 0$.

¹ The hydrogen ion concentration in blood is very low in comparison to that of the gastric acid and can be neglected ($= 0$).

For the solution of the differential eqs. (33) and (36) it is of no importance whether C_0 is a constant or not. The main thing is that it is constant for one single v value. Therefore we may substitute Pa for C_0 . Eq. (35) will then be

$$H = \frac{P_\infty + (P_0 - P_\infty) e^{-k,v}}{\frac{k}{v} + 1} \quad (39)$$

The curve corresponding to this equation is roughly similar in shape to that given by (35), as for $v=0$, $H=0$, and for $v=\infty$, $H=P_\infty$.

Furthermore the derivatives *at the origin* ($v=0$) are:

for (35)

$$\frac{dH}{dv} = \frac{C_0}{k} \quad (40)$$

and

$$\frac{d^2H}{dv^2} = -\frac{2C_0}{k^2} \quad (41)$$

and for (39)

$$\frac{dH}{dv} = \frac{P_0}{k} \quad (42)$$

and

$$\frac{d^2H}{dv^2} = -\frac{2P_0}{k^2} \quad (43)$$

With the constants involved, the general shapes of the two curves will be very similar. As, however, P_0 is greater than C_0 the curve for the *Sa* will rise more quickly (cf. eqs. (40) and (42)).

In conclusion it may be said that it does not matter whether the *Pa* is constant or not, as the general shape of the curve relating the *Sa* to the secretion rate will be almost the same in both cases.

Experimental data.

In *fig. 22* the relation between the secretion rate and the secondary acidity is given. Every point in this curve is obtained from several determinations of a steady state level of the secretion rate and the corresponding acidity value (cf. *fig. 12*, page 27). (Similar curves from other dogs are found in *fig. 25*, page 60). The significance of these results will now be discussed.

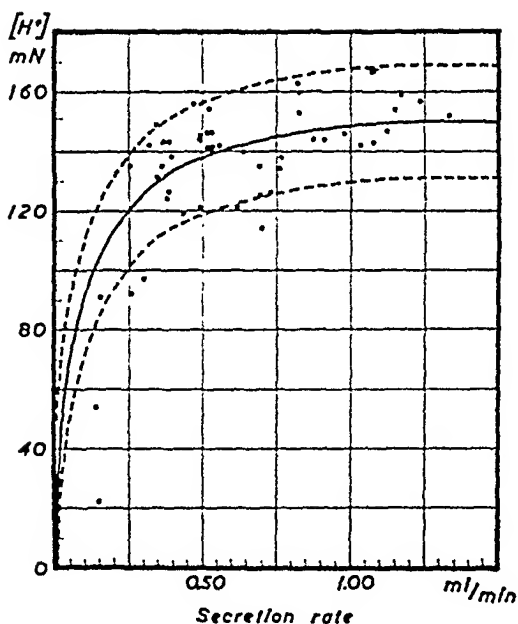


Fig. 22. Relation between the secondary acidity and the secretion rate (dog S). The full line is the curve derived from the diffusion theory which best fits the actual observations. The dotted lines denote the "standard error of estimate."

In the foregoing pages the factors that may reduce the strength of the *Pa* have been mentioned, they are:

- 1) *Dilution*,
- 2) *Neutralization* and
- 3) *Diffusion*.

No other possibilities appear to exist. Every factor mentioned in the literature can be included in one or more of these three categories. It should be pointed out, that diffusion is used in such a wide sense, that adsorption under special conditions may be described as diffusion. The reason is, that in the technique used (see chapter 2) an adsorption that will load the mucosa or its mucus lining gives rise to no problem as the results discussed are not observed until a convenient time has elapsed and the secretion rate and acidity have become constant. That means that the mucosa is already loaded and can no longer cause a further adsorption. If, however, the adsorbed amount should be given off on the other side (the blood side or nutrition side) more acid could be adsorbed from the gastric contents. The remaining process will then be analogous to that of diffusion.

If on the other hand a continuous secretion of mucus should occur thus adsorbing acid during the entire experiment, this mucus secreted

would be obtained in the measuring cylinder (a continuous accumulation of mucus within the pouch would be impossible). In the present experiments the acidity values, which will be discussed, are determined as the total acidity (titration with brom-thymol-blue pH range 6.0—7.6 cf. chapter 3, page 15) and therefore any acid which may have been adsorbed or neutralized by mucus has also been detected. Only a very small amount can still be fixed to mucus according to the acid-base binding curves for mucus made by IHRE (1938).

Thus there remain only the three possibilities mentioned above: dilution, neutralization and diffusion.

1. *Dilution.*

Every non-acid admixture to the stomach contents acts as a diluent. Examples may be found in test meals or washing fluids. In the present experiments no test meal is used, but the pouch is washed out with saline before every experiment. During the first minutes of an experiment this will of course dilute the secreted acid, but the effect will be *more and more insignificant as the saline is forced out, and when the acidity has become constant we have reason to believe that it has no more influence.*

Those components of the gastric secretion which are non-acid may also act as diluents. Such constituents are *mucus*, *enzymes* and "*diluting secretion*" — "*Verdünnungssekretion*" — (if such a secretion does exist).

As the main question is whether there is something secreted which may dilute the parietal secretion, it is of no importance to know if this is mucus, enzymes or some other "*Verdünnungssekretion.*" Therefore we call all these *diluting secretions*.

The diluting secretions.

Very few authors have tried to prove the existence of a diluting secretion after histamine stimulation — cf. HOLLANDER & COWGILL (1931), ENGSTRÖM (1935) and HOLLANDER (1938).

Experiments concerning the primary acidity by LINDE, TEORELL & ÖBRINK (1947) seem, however, to disprove the existence of any important diluting secretion.

By carefully maintaining constant secretion rates they were able to prove that the difference between the primary and the secondary acidity was not due to any diluting admixture to the primary acid.

Only at extremely low secretory rates, less than 0.01 ml/min, a slight diluting secretion was significant. The order of such a secretion would be about 0.01 ml/min, from which it follows that dilution would be significant only for secretion rates of the order of 0.01—0.05 ml/min (vide infra).

It might be stated that in the present experiments *any significant diluting secretion can be excluded* with the exception of this unimportant amount of non-acid admixture.

2. Neutralization.

The only constituents of the gastric juice that could have any neutralizing effects would have to contain some basic radicals. Such are present in the proteins and in the mucus owing to their ampholytic character. The acid-binding capacity of mucus is very slight according to IHRE (1938). The concentration of bicarbonate in the mucus has been determined with the following results: BOLTON & GOODHART (1931) — 39 mN; WEBSTER (1933) — 16.8 mN; BAXTER (1934) — 11.2—14.0 mN, IHRE (1938) — 3.2—10.8 mN and MAHLO (1938), who considers the mucus to have an acid reaction.

The bicarbonate reacts with the hydrochloric acid to form sodium chloride and carbonic acid, which in a second step would give water and carbon dioxide. This gas would easily be re-absorbed or given off into the air and thus escape a determination.

In this connection it should be pointed out that such a neutralizing effect may also have occurred in the estimation of the primary acidity — LINDE, TEORELL & ÖBRINK (1947) — and is therefore included in the calculated value of the Pa .

If, in spite of the above, the mucus should lower the acidity could it be responsible for the acidity decrease in fig. 22? A secretion rate of 0.06 ml/min corresponds to an acidity value of 38 mN. That means a decrease of 132 mN from a constant C_0 -value of 170 mN. If the mucus were responsible for this and is considered to contain 10 mN of alkali, its volume-rate (m ml/min) can be calculated in the following way:

$$(0.06 - m) \times 170 - m \times 10 = 0.06 \times 38$$

or

$$m = 0.04 \text{ ml/min.}$$

At such a secretion rate it would also act as a diluting fluid, which

has been disproved above (page 52). If the secretion of mucus should take place within probable limits (0.01 ml/min) the alkaline strength of it (n mN) would be

$$0.05 \times 170 - 0.01 \times n = 0.06 \times 38$$

or

$$n = 622 \text{ mN,}$$

which is in absolute disagreement with earlier investigations.

Thus, it may be stated that *the effect of the mucus in regulating acidity can generally be neglected.*

This view is contrary to that of many investigators. BABKIN and his collaborators have made many experiments showing the influence of the mucus. Here it would be of interest to discuss one paper of GRANT (1942) which is also referred to by BABKIN (1944 page 406).

GRANT introduced hydrochloric acid into a cat's stomach and observed the acidity decrease, but in order to prove that the decrease is dependant upon the neutralizing power of the mucus she collected mucus after stimulation with 1 % acetic acid or 70 % alcohol. This mucus was exposed to 130 mN HCl *in vitro* for 15 minutes. The acidity decrease was determined as well as the calcium uptake from the mucus, which always contains Ca (GRANT 1941). After this period a fresh volume of 130 mN HCl is mixed with the same mucus and the procedure is repeated three times.

The result is an acidity reduction which is most significant in the first, less in the second and least in the third period. The calcium uptake shows the same relations. *Table 4* gives the data.

Even so the neutralizing power of the mucus can be doubted. The calcium uptake from the mucus may be a matter of diffusion. The hydrogen ions could also diffuse into the mucus.

As we know from *Table 4* to what degree Ca^{++} has diffused out from the mucus we can calculate the amount of H^{+} -ions, which have entered the mucus in the same time. The calculated acidity reduction is given in column 8 *Table 4* and shows that the total decrease is *almost entirely a matter of diffusion*. Only the small differences between the observed and the calculated values may be a result of the bicarbonate content of the mucus. This is a quite different explanation than that offered by GRANT.

Table 4. *The neutralizing capacity of mucus. (Columns 1—7 from GRANT (1942), columns 8—9 are calculated by the author.)*

| Cat No. | Period of 15 min | Volume of 130 mN HCl in contact with the mucus ml | Volume of the mucus ml | Calcium in mucus mg/100 ml | Calcium uptake mg/100 ml | Acidity reduction observed mN | Acidity reduction calculated mN | Difference mN |
|---------|------------------|---|------------------------|----------------------------|--------------------------|-------------------------------|---------------------------------|---------------|
| 2 | 1 | 7.0 | 3.0 | 6.00 | 0.90 | 40 | 33 | + 7 |
| | 2 | | | | 0.65 | 15 | 14 | + 1 |
| | 3 | | | | 0.45 | 6 | 6 | 0 |
| 3 | 1 | 7.2 | 3.6 | 10.10 | 1.85 | 46 | 38 | + 8 |
| | 2 | | | | 1.15 | 24 | 14 | +10 |
| | 3 | | | | 0.70 | 6 | 7 | - 1 |
| 4 | 1 | 7.0 | 5.2 | 10.48 | 1.60 | 56 | 38 | +18 |
| | 2 | | | | 1.30 | 29 | 24 | + 5 |
| | 3 | | | | 0.80 | 11 | 12 | - 1 |
| 5 | 1 | 7.0 | 3.3 | 8.75 | 1.40 | 37 | 36 | + 1 |
| | 2 | | | | 1.15 | 20 | 16 | + 4 |
| | 3 | | | | 0.80 | 8 | 6 | + 2 |
| 6 | 1 | 7.0 | 4.5 | 7.37 | 1.65 | 53 | 46 | + 7 |
| | 2 | | | | 1.30 | 28 | 22 | + 6 |
| | 3 | | | | 0.60 | 14 | 8 | + 6 |

The secretion rate of mucus.

It would be of great interest to know how much mucus is secreted during an experiment. This demands a method of determining the mucus, but specific methods do not exist at present. Many suggestions have been made, and in the present paper four methods have been used.

A) Titration curves: The main question is to what extent the mucus can neutralize the acid. This might be detected by making an ordinary titration curve of the acid-binding capacity.

Many such curves were made on samples of gastric juice from the pouch dogs, but none showed any properties that could originate from the mucus. Every curve was perfectly flat.

B) Calcium analyses: GRANT (1941, 1942, 1944) states that the content of calcium should be directly proportional to the acidity decrease and to the amount of mucus, and therefore Ca has been

Table 5. *Calcium in the histamine induced gastric secretion.*

| Secretion rate
ml/min | Time after
starting the
experiment
hours | Calcium con-
centration
mg/100 ml |
|--------------------------|---|---|
| 0.10 | 0 ⁵⁰ | 1.66 |
| | 1 ²⁰ | 0.25 |
| | 1 ⁵⁰ | 0.00 |
| | 2 ¹⁰ | 0.00 |
| | 2 ³⁰ | 0.00 |
| | 2 ⁵⁰ | 0.00 |
| 0.81 | 0 ²⁰ | 0.77 |
| | 0 ⁵⁰ | 0.38 |
| | 1 ¹⁰ | 0.00 |
| | 1 ²⁰ | 0.00 |
| | 2 ⁰⁰ | 0.00 |

determined. The results from one dog are found in *Table 5*. There it may be seen that in the first few periods of 10 minutes some Ca is detectable but it then disappears altogether from the gastric juice. *The mucus (as Ca) seems to have been washed out.*

C) Nitrogen analyses: Mucus is a protein-like substance and will thus be detectable by nitrogen analysis. These values also decrease during an experiment as if a washing-out effect existed. Yet, nitrogen remains present during the whole experiment, and some part of it may originate from a protein. If *all* the nitrogen is considered to originate from proteins a rough estimate of the acid-binding capacity would be obtained by a titration curve of another protein mixture of a known strength.

A suspension of hen's egg-white containing 1 mg of nitrogen per ml has been used. The result is given in *fig. 23*, which shows the base-acid-binding capacity of 5 ml of the protein solution expressed in ml of a tenth normal (N/10) of a proteolyte. Comparing this with *Table 6* and *fig. 24* where the nitrogen content of the gastric secretions are plotted against the secretion rate, it is easy to understand the relatively low acid-binding capacity of the juice.

Consequently, *this mode of determination also indicates the very slight effect of the mucus upon the hydrochloric acid.*

D) Reduction power analyses: The mucus is considered to be a mucoprotein and will consequently contain a hexose group. It will therefore show a *reduction power* after hydrolysis.

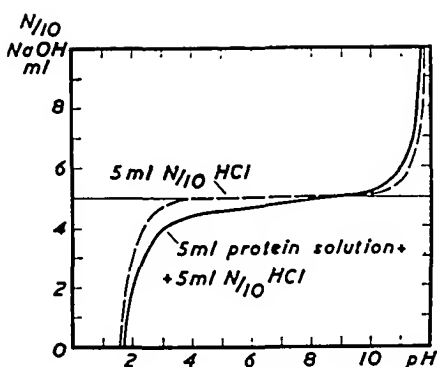


Fig. 23. The acid-base-binding capacity of a protein solution (hens egg-white) containing 1 mg nitrogen per ml.

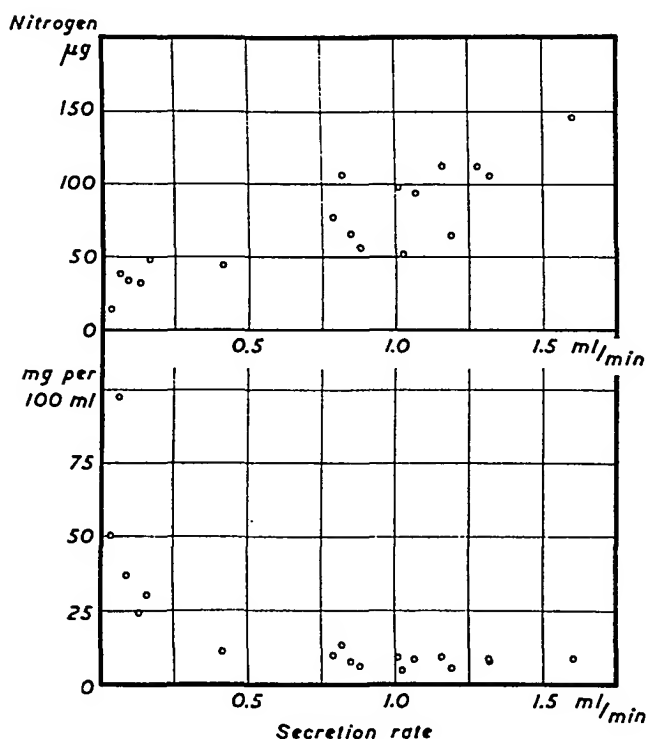


Fig. 24. The concentration and total amount of nitrogen in relation to the secretion rate.

The results are expressed in mg glucose per 100 ml juice and are seen in Table 7. IHRE (1938) who investigated almost pure gastric mucus found 191 mg/100 ml. That means that the present mucus is diluted $> 1:20$. The volume of this mucus would then be in the order of 0.01 ml.

Table 6. *The pepsin content and N-concentration in the gastric juice (cf. fig. 24).*

| Secretion rate
ml/min | Pepsin
mg protein/ml/min | Nitrogen
mg/100 ml |
|--------------------------|-----------------------------|-----------------------|
| 0.06 | — | 98.0 |
| 0.16 | 1.23 | 30.0 |
| 0.41 | — | 10.9 |
| 0.79 | — | 9.8 |
| 0.82 | 1.45 | 13.1 |
| 0.85 | 0.64 | 7.7 |
| 0.88 | 0.47 | 6.2 |
| 1.01 | 0.89 | 9.7 |
| 1.02 | 0.73 | 5.0 |
| 1.07 | 0.98 | 8.8 |
| 1.16 | 0.83 | 9.8 |
| 1.19 | 0.57 | 5.4 |
| 1.28 | 0.78 | 8.8 |
| 1.32 | 0.51 | 8.0 |
| 1.60 | 0.77 | 9.1 |

Table 7. *The reducing power (glucose) of histamine induced gastric juice.*

| Secretion rate
ml/min | Reducing power
mg glucose/100 ml |
|--------------------------|-------------------------------------|
| 0.24 | 8.3 |
| 0.33 | 6.3 |
| 0.62 | 7.6 |

It is the opinion of the author, that all these experiments together give support to the view, that *the acid-binding capacity of the mucus after stimulation with histamine is of very little importance.*

The pepsin content of the histamine induced gastric juice.

It might be of interest to know the amount of pepsin secreted by the present stimulation method, as a large concentration might be responsible for an acidity decrease.

From the titration curves related above, we cannot expect to find any considerable amounts as the pepsin ought to show up in these experimental curves as well as the mucus.

Unlike the mucus, pepsin is, however, rather easy to determine and the amount present has been analyzed when the secretion rates have been held constant. It is expressed in mg protein that has been digested by 1 ml gastric juice during 1 minute (cf. Methods, page 16).

The results are collected in Table 6, where we also see the nitrogen content of the same samples.

As expected, the values are rather small and seem to be independent of the secretory rate and the stimulation. This is in accordance with BJÖRKMAN, NORDÉN & UVNÄS (1943) who consider that histamine does not stimulate or inhibit pepsin secretion.

In a few cases the peptic power runs parallel to the nitrogen concentration. As mentioned above this non-parietal secretion can only be of very slight importance in the acidity regulation.

3. Diffusion.

The third factor responsible for the acidity decrease will now be discussed. The first two have been considered to be of negligible importance and thus the diffusion may be the main factor. How do the experiments agree with the theoretical considerations from the diffusion theory?

One of the best tests for such a question is to calculate the correlation between the theoretical equation and the experimental data.

Eq. (35)

$$H = \frac{C_0}{\frac{k}{v} + 1}$$

may be transformed to

$$vC_0 - Hk = Hv. \quad (44)$$

By using the methods of least squares we obtain the following normal equations:

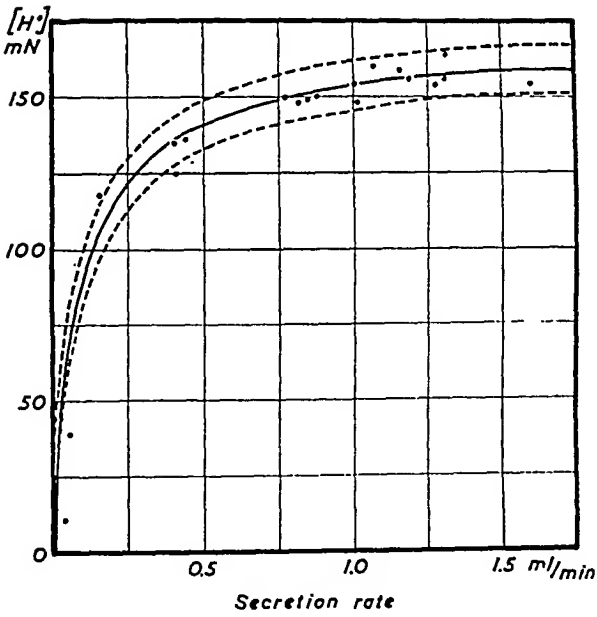
$$\begin{cases} C_0 \sum v^2 - k \sum Hv = \sum Hv^2 \\ C_0 \sum Hv - k \sum H^2 = \sum H^2 v. \end{cases} \quad (45)$$

(46)

Solving this is a matter of routine and will be omitted here. The results for three dogs are collected in Table 8 and Fig. 22 and 25.

In these figures eq. (35) is drawn as full lines and the limits within \pm the standard error of estimate as broken ones. The agreement be-

Dog B



Dog R

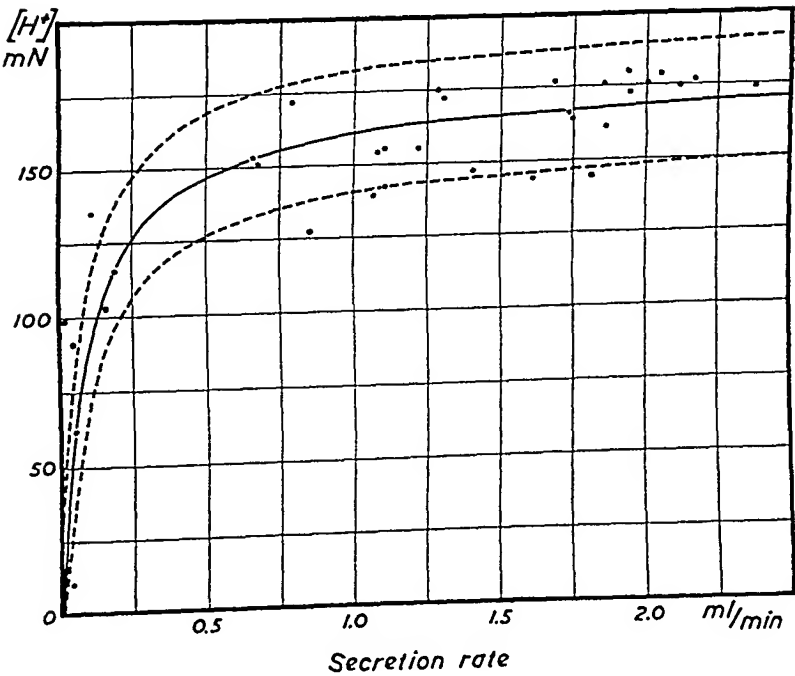


Fig. 25. Relation between the secondary acidity and the secretion rate.

tween the theoretical function and the experimental data is good. Only at the lowest secretion rates is the actual acidity somewhat lower than the calculated one. This may partly be due to experimental errors but here the small amount of diluting fluid, which can never be excluded, and which has been discussed above, may have had some influence.

Table 8. Statistical data to fig. 22 and 25.

$$\text{Regression line: } H = \frac{C_0}{\frac{k}{v} + 1}$$

(H = secondary acidity, mN; C_0 = primary acidity (a constant), k = permeability coefficient (a constant) and v = secretion rate, ml/min)

| | Dog S
(n = 47) | Dog R
(n = 33) | Dog B
(n = 19) |
|----------------------------|-------------------|-------------------|-------------------|
| C_0 (mN) | 160 ± 5 | 177 ± 6 | 168 ± 1 |
| k (ml/min) | 0.08 ± 0.02 | 0.10 ± 0.06 | 0.10 ± 0.02 |
| Standard error of estimate | ± 19 | ± 21 | ± 8 |
| Index of correlation | 0.70 ± 0.08 | 0.83 ± 0.06 | 0.98 ± 0.01 |

(n = number of observations)

We ought, however, to suspect that in the experiments with these low secretory rates the "washing out" of mucus and enzymes, which have collected during the hours preceeding the experiment has been ineffective. Therefore, in a few cases, the pouch has been washed out with 170 mN HCl (instead of saline) before starting the histamine injection. This different procedure gave, however, the same result indicating that all the washing fluid was poured out and no acid-binding capacity was left in the pouch to cause the low acidity values. This must therefore be a result of a very slight dilution superimposed on the main factor — diffusion.

Influence of time.

There are some special investigations, which ought to be considered. From the eq. (35) it is suggested that time is of no significance in the acidity reduction, i. e. *the mode of sampling is of no importance*. The present experiments are usually carried out by letting the juice flow out of the pouch continuously, and therefore controls have been made

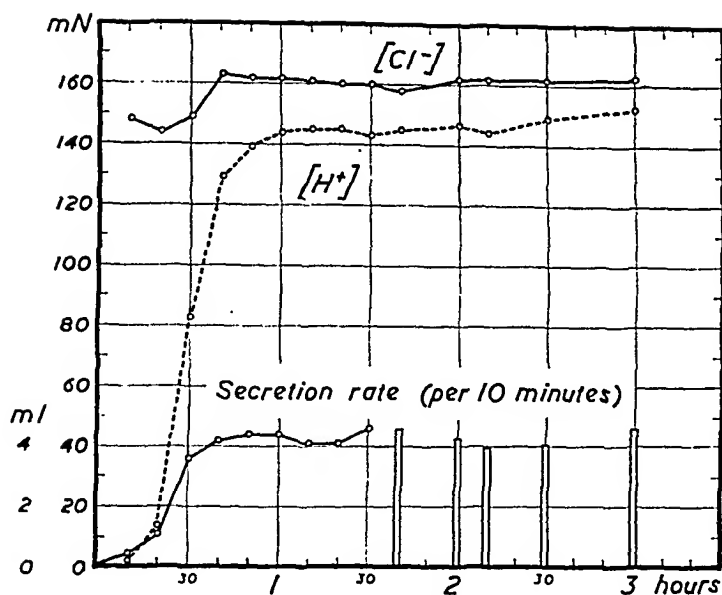


Fig. 26. Experiment showing the insignificance of mode of sampling on the acidity and the chloride. Between 0⁰⁰—1³⁰ hrs there was a continuous outflow from the pouch. After 1³⁰ the collection was intermittent (10, 20, 10, 20, 30 minutes).

in which the juice was recorded in an intermittent way. Fig. 26 shows the results. The acidity values were the same after a continuous and an intermittent collection. Any occlusion of the pouch was of no importance, i. e. *the time is insignificant for the acidity reduction* — cf. GUDIKSEN (1943), TEORELL (1947). This invalidates the objections by WELIN & FRISK (1936) and BABKIN, HEBB & KREUGER (1941) that normally there cannot be sufficient time for diffusion to occur.

Apparent inconsistencies of the diffusion theory.

In some papers some inconsistencies have been remarked, that tend to invalidate the hypothesis of diffusion. One of these is the observation made by PAVLOV, that the acidity at the beginning of an experiment was very low and increased when the secretion rate increased. At the end of an experiment, however, the decrease in the secretion rate was not followed by a similar decrease in the acidity. In the present experiments the same thing has occurred. How can that possibly fit in with the diffusion theory? In fact, these circumstances have been one of the things which have been most difficult to explain.

The experiments with steady state values, however, made it clear that the relation between secretion rate and acidity is very close.

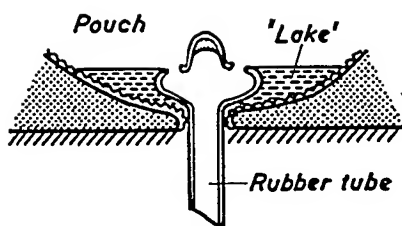


Fig. 27. The position of the Pezzer catheter in the pouch.

There must be some explanation for the inconsistency mentioned and it might be the following:

The "lake"-hypothesis. Every attempt to empty *completely* a stomach or a pouch is unsuccessful. There will always be some volume left. This is true of present experiments too. From *fig. 27* the position of the Pezzer catheter in the pouch is seen. In addition to the volume resting in crypts and elsewhere on the mucosa a kind of a "lake" is formed around the catheter (see the *fig.*). The freshly secreted juice runs into this "lake" and some part of the content of the "lake" is drained through the tube.

Experiments have been carried out to determine the size of this "lake" with phenol red (for the method see p. 18), and for one of the dogs the average volume determination was 4.0 ml. This very dog showed the typical curves of the acidity described by Pavlov as related above. One of them is seen in *fig. 28* (curve 1).

The "lake" causes a *time lag* in the sampling of the juice. If we know the secretion rate and the size of the "lake" and if the secreted volume drives the "lake" out entirely before it enters the tube, and if *no mixing* of the secreted volume and the "lake content" takes place, we are able to calculate this time lag.

When the secretion rate is constant and everything else is also constant this time lag is of no importance. This can be shown by putting $t = \infty$ in eq. (34). It does not matter whether two observed values are coincident or not as no change occurs during the time. In the case of a varying secretion rate, however, as in the beginning and at the end of an experiment it will be most necessary to take the time lag into account.

In *fig. 28* the typical curve shows that the acidity does not decrease at the end of the experiment (curve 1), as might be expected from the diffusion theory (curve 2). If, however, the time lag is considered, a curve more like the actual one is obtained (curve 3).

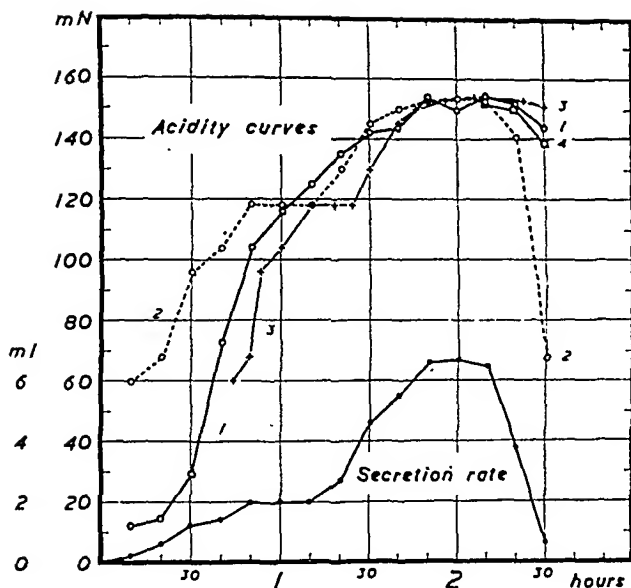


Fig. 28. A typical experiment with a continuous secretion. (Two secretion rates were established.) The acidity curve (1) did not decrease at the end of the experiment as expected from the diffusion theory (2). If this curve (2) is corrected for the effects of the "lake" (see fig. 27) one obtains the curves (3) or (4). (See text.)

If in addition to the above a *complete mixing* occurs between the content in the "lake" and the secreted amount, a somewhat different result will occur. This offers a much more complicated problem but may be solved in the following way:

Consider the secretion rate to be constant under a 2-minutes period (v ml/min): The secreted acid has a secondary acidity of H_1 mN. The "lake" contains p ml and at the beginning of the period its concentration is H_0 mN.

I) First consider the concentration changes in the "lake":

$$\text{increment due to secretion} = \frac{v H_1}{p} dt \quad (47)$$

$$\text{decrement due to dilution} = -\frac{v dt}{p} \cdot H \quad (48)$$

This makes the concentration increase

$$dH = \frac{v(H_1 - H)}{p} \cdot dt \quad (49)$$

The solution gives

$$H = H_1 - (H_1 - H_0) e^{-\frac{vt}{p}} \quad (50)$$

II) The amount of hydrochloric acid (M m.eq.) driven through the catheter is calculated from

$$\int_{t=0}^{t=2} dM = \int_{t=0}^{t=2} v H dt \quad (51)$$

which gives

$$M = 2 v H_1 - p (H_1 - H_0) (1 - e^{-\frac{2v}{p}}). \quad (52)$$

When the amount has been calculated according to this formula we start with a new 2-minute period with a new v -value but with H_0 substituted by the H -value in (50) which corresponded to $t = 2$ min. So the calculations may continue for five periods. If the total amount for these ten minutes is divided by the total volume we get the concentration in the 10 minute sample. This procedure led to the construction of the curve 4 in fig. 28. This curve has been calculated only for the descending part of the experiment.

In reality the postulate of "no mixing" and "complete mixing" in the "lake" are extremities, the truth lying somewhere in between.

This "lake-theory" was supported by the following experiments: At the end of an experiment when the last sample was obtained, a small pipette was introduced into the pouch and brought in contact with the mucosa in the upper part of the pouch. A small volume of juice which collected in the pipette showed an acidity value which was considerably lower than that of the last sample obtained from the "lake" contents. Results like the following were obtained (the first number is the last "lake" acidity value, the one in brackets is the corresponding "pipette-value"): 139 (105) mN, 135 (25) mN, 116 (58) mN.

We have now seen that the apparent inconsistency in the acidity curve can be explained by the existence of a "diluting" volume, which we have called the "lake".

That this "lake" is insignificant in the steady state values of the acidity even at very low secretory rates was shown in the experiment related above, where a washing with 170 mN HCl instead of NaCl gave the same constant acidity value.

Thus the continuous intravenous technique has undoubtedly shown that *a definite relation exists between the secretion rate and the secondary acidity.*

A conclusion, that can be drawn from the above is that *the diffusion process takes place at the very spot where the acid was secreted. The time required for the juice to run along the mucosal surface does not influence the resulting acidity* (cf. page 61).

Such a consideration is supported by an experiment, in which the dog was laid on its back during the experiment. In the erect position one part of the surface where the juice collects is formed by the head of the rubber tube (cf. fig. 27), where no diffusion can take place. When the dog is in a recumbent position, however, the "lake" will be in contact only with a mucous membrane. In spite of this the acidity was uninfluenced by the arrangement. The result was expected and in accordance with the diffusion theory.

Resorption of hydrogen ions in the non-secreting pouch.

In a whole stomach pouch of a cat TEORELL (1933) found an acidity decrease of an instilled isotonic solution of HCl (170 mN). He ascribed this phenomenon to a diffusion of the hydrogen ions through the gastric mucosa. IHRE (1938), ELLIOT, RISHOLM & ÖBRINK (1942) confirmed his results on adults and KAIJSER (1943) on children. In stomach pouches of dogs BOLDYREFF (1934) failed to get any decrease in acidity of an instilled acid. He, however, used very large volumes (75 ml) which made the influence of the diffusion mechanism insignificant. This was also pointed out by TEORELL (1935 a) (*vide infra*).

The present author has made the same instillation experiments as Boldyreff but with much smaller volumes (10, 15 and 20 ml). In fig. 29 a typical experiment on dog R is seen. The acidity decrease is almost exponential (cf. fig. 29 a) and could be expressed

$$H = H_0 e^{-\frac{ct}{p}} \quad (53)$$

where H = the acidity in the pouch, H_0 = the initial concentration of the instilled acid, t = time (in minutes), c = the permeability coefficient (ml min^{-1}) and p = volume (ml).

The value of $\frac{c}{p}$ can be determined from the slope of the curve in fig. 29 b. It is $\frac{c}{p} = 0.013 \text{ min}^{-1}$.

By multiplying this by the average volume in the experiment (8 ml) one gets the permeability coefficient $c = 8 \times 0.013 = 0.10 \text{ ml min}^{-1}$.

This may be compared with the permeability coefficient in eq. (35), and Table 8 dog R, which is also 0.10 ml min^{-1} .

These findings are further support for the theory of diffusion as a main factor in the acidity regulation.

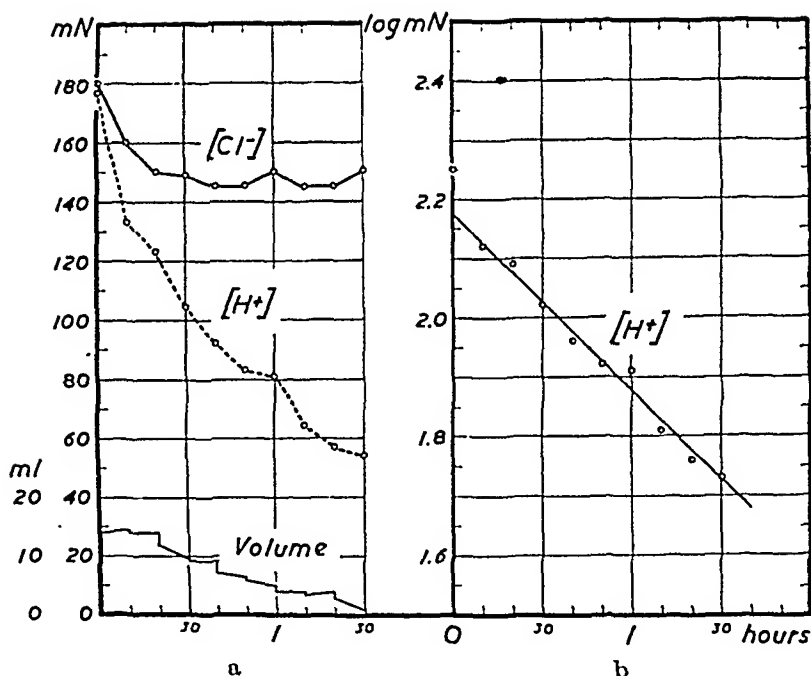


Fig. 29. An instillation experiment in a cat's stomach of 170 mN HCl. a) The experimental results. b) The logarithm of the acidity values. The slope of this free hand line is expressed by the "permeability coefficient" (cf. eq. 53).

According to the formula (53) one can easily calculate what would happen in the above mentioned dog R if 75 ml of 170 mN HCl were introduced in the pouch as in Boldyreff's experiments. (In fact such a large volume would cause a severe distension of the pouch.)

After 60 minutes we should obtain

$$H = 170 \cdot e^{-\frac{0.10 \cdot 60}{75}} = 169 \text{ mN,}$$

i. e. the acidity decrease would scarcely be detectable and consequently the findings of Boldyreff do not contradict the diffusion theory.

CHAPTER 11.

Total Output of Hydrochloric Acid in Relation to the Secretion Rate.

Theoretical considerations.

A computation of the total amount of HCl obtained from a gastric pouch after histamine stimulation leads to some interesting conclusions, which will be described here.

When the secretion is going on at a *constant rate* the eq. (35) is valid

$$H = \frac{C_0}{\frac{k}{v} + 1}$$

The total amount will therefore be

$$vH = \frac{v \cdot C_0}{\frac{k}{v} + 1} = vC_0 \left(\frac{1}{1 + \frac{k}{v}} \right) \quad (54)$$

which could be transformed to

$$vH = vC_0 - k \left(\frac{C_0}{\frac{k}{v} + 1} \right) \quad (55)$$

or by substituting (35)

$$vH = vC_0 - kH \quad (56)$$

The expression vC_0 is the HCl secreted per minute and kH is the amount of hydrogen ions diffusing out of the stomach. The difference ($vC_0 - kH$) is the HCl actually obtained. Graphical representations of these functions, "*HCl secreted*", "*H⁺ diffused*" and "*HCl obtained*" are given in *fig. 30*.

Putting $\frac{k}{v} \ll 1$ in eq. (54) one can approximate

$$\frac{1}{1 + \frac{k}{v}} = 1 - \frac{k}{v} \quad (57)$$

A transformation of (54) will therefore give

$$vH = vC_0 - kC_0 \quad (58)$$

The higher the secretion rate the better the approximation. For a k -value of 0.10 and $C_0 = 170$ mN the following differences between eq. (56) and (58) in microequivalents/min will appear:

| | | | |
|-------------------|-------|-------------------|----------|
| $v = 0.00$ ml/min | — | difference = 17.0 | μeq./min |
| $v = 0.10$ | » — » | = 8.5 | » |
| $v = 0.20$ | » — » | = 5.8 | » |
| $v = 0.50$ | » — » | = 2.8 | » |
| $v = 1.00$ | » — » | = 1.7 | » |

When $v > 0.20$ the approximation will be quite justifiable. The actual experimental values lie in general far above this limit.

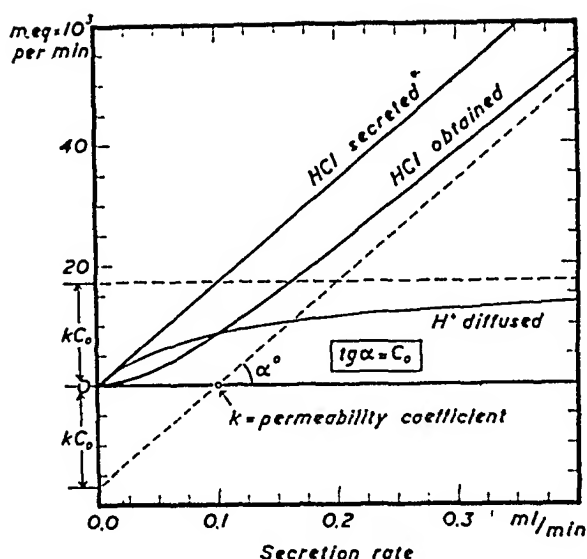


Fig. 30. The theoretical behaviour of the total amount of HCl obtained in relation to the secretion rate. It approaches the oblique dotted line which has the mathematical expression $vH = C_0 v - kC_0$. (See text.)

As vH and v are the only variables, the equation (58) is expressed by a *straight line*, where v is the independent and vH the dependent variable, C_0 is the differential factor for the function and kC_0 is the intercept on the vH -axis. The v -intercept will equal k . The meaning of eq. (58) is explained by the *fig. 30* (the oblique dotted line).

Here we ought to call to mind eq. (38) (page 49)

$$H = C_0 \left(1 - \frac{k}{v} \right)$$

which was valid for the case where the *diffusion through the mucous membrane was proportional to the primary acidity itself*, i. e. no mixing of the acid as secreted and the contents of the stomach was possible. Calculating the relation between the total amount and the secretion rate according to this formula the eq. (58) appears *without* the use of any approximation. It was the finding of such a linear relationship between the secretion rate and the output of HCl that made GRAY, BUCHER & HARMAN (1941) believe that the relation of acidity to secretion rate followed the hyperbolic curve expressed by eq. (38). (Cf. page 49.)

Experimental data.

The relation between the total acid output and the secretion rate is seen in *fig. 31*. (The results are obtained from dog B.) The points are smoothed with a straight line (freehand) corresponding to the eq.

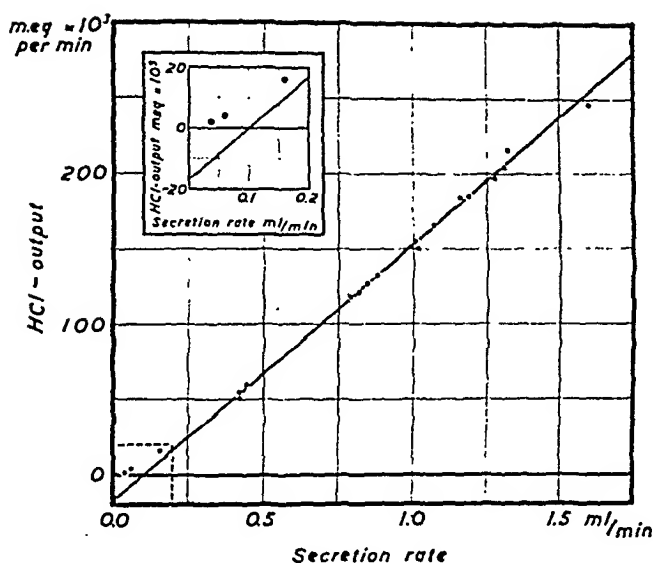


Fig. 31. The total amount of HCl in relation to the secretion rate (cf. fig. 30). The dotted square in the lower left-hand corner is enlarged in the inset.

(58). It is seen that the values are very well fitted by this regression line. C_0 , i. e. the slope of the line is 170 mN and $k = 0.10$ ml/min. These values agree quite well with those given in Table 8 (page 61).

Only for the lowest secretory rates is there a divergence between the points and the line, indicating that the real experimental curve follows the non-approximated eq. (56) instead of (58). Accordingly *the diffusion from the stomach is proportional to the actual hydrogen ion concentration and not to the primary acidity.*

From this statement it follows that the extrapolation curve given by GRAY, BUCHER & HARMAN (1941) for the relation between secretion rate and acidity (cf. p. 49) must be an approximate one, and that the experimental data are in better accordance with the diffusion formula (35), page 48.

CHAPTER 12.

The Chloride Concentration in the Gastric Juice.

Although the acidity has been the most favoured subject for gastric research, the chloride concentration offers just as many problems which are unclear and baffling. A knowledge of the behaviour of the chloride ion would perhaps be valuable in the general discussion of HCl formation.

When reading papers dealing with the problems involved here one very soon finds, that the experimental data are contradictory and vague. Several investigators have found a close relation between the acidity and the chlorides; among them BOLTON & GOODHART (1931), APPERLY & CRABTREE (1931), HOLLANDER (1932), TEORELL (1933), NORDENFELT & TEORELL (1935), WELIN & FRISK (1936), IHRE (1938), GRAY & BUCHER (1941), GRAY, BUCHER & HARMAN (1941), HORSTMAN (1946, 1947); among those who have not ROSEMAN (1907) and BERGLUND, JOHNSON & CHANG (1935).

With the technique used in this paper there is an opportunity to find the real correlation between the chloride concentration and the secretion rate.

Analogous to the acidity determinations the chloride concentrations are expressed in millinormality (mN).

The chloride concentration in the "primary secretion".

From the results of LINDE, TEORELL & ÖBRINK (1947) it may be seen that the Cl values in the "primary secretion" were as high as the acid concentration, and even higher. This is an indication that the acid was secreted as HCl even at the greatest concentrations. As mentioned above (see Chapter 9 page 46) the presence of the isotonic glycine buffer, in which the secreted juice was collected, might have prevented the hypertonicity of such a strong acid. This may possibly be the reason for the high values obtained, and because of the uncertainty at present it seems better to assume a constant primary acidity in the following (cf. page 46).

The "*primary chloride*" will therefore be considered to have a constant concentration of C_0 mN (cf. page 48).

The regulation of the chloride concentration.

The relations between the secretion rate and the "*secondary chloride*" concentration from the present experiments are found in *fig. 32* (the secondary acidity curve is plotted as a dotted line).

It is at once clear that the chloride never followed the low acidity values, but, on the other hand, it showed a certain tendency to decline when the secretion rate was decreasing. As in the case of the acidity, *some reduction of the chloride concentration has occurred though to a lesser extent.*

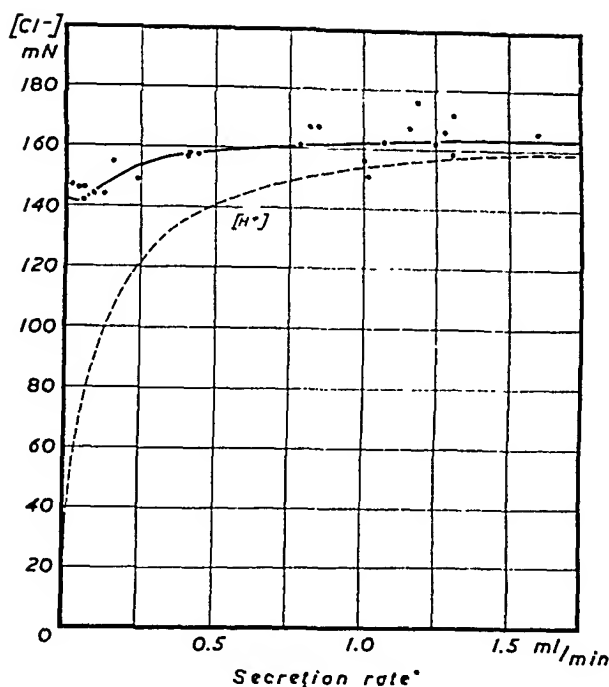


Fig. 32. The relation between the chloride concentration and the secretion rate. The experimental data are fitted with eq. (65) by trial and error. $S_0 = 142$ mN; $r = 0.07$ ml/min; $C_0 = 168$ mN; $k = 0.10$ ml/min. (Dog B.)

The three factors acting as acidity reducers may be discussed also when dealing with the chlorides:

1. *Dilution* by a non-acid admixture will of course have the same influence on the chloride concentration as on the acidity, presuming that it is chloride free. However, no diluting fluid in the stomach can be expected to be free from chloride, and this leads us to investigate the possibility of dilution.

2. "Neutralization" of chloride is not known to occur in the stomach.

3. *Diffusion* of chloride is very probable, though there are authors who deny any migration of chloride ions through the gastric mucosa — GILMAN & COWGILL (1933).

Dilution.

If the main factor in the acidity reduction were an admixture with mucus, this should affect the chloride concentration similarly. The chloride content of the mucus is considered to be 95 mN — VINEBERG (1931) — and would consequently diminish the chloride content of the acid. Some higher chloride values are given by WEBSTER (1933) and BAXTER (1934).

By the present technique it has been possible to obtain low secretion rates with very low acidity concentrations. In these cases the chloride values remained almost constant. This invalidates the theory of dilution by mucus. According to this we should have expected values of about 95—100 mN Cl^- for a secretion rate approaching zero. Another diluting fluid rich in chloride would of course afford a possible explanation, but doubt has already been cast on the presence of a diluting secretion for other reasons (cf. page 52).

Diffusion.

There remains diffusion to be discussed. In an instilled volume of HCl (170 mN) in a tied-off cat's stomach the chloride is lowered to 140—150 mN — TEORELL (1933, 1939, 1940). This is also valid for human beings — IHRE (1938), ELLIOT, RISHOLM & ÖBRINK (1942), KAIJSER (1943). A diffusion of chloride through the gastric mucosa is also postulated by SHAY, KOMAROW, SIPLET & FELS (1946).

The total chloride is the sum of the acid chloride (which is of the same magnitude as the secondary acidity) and the neutral chloride. The latter may be considered as sodium chloride (in reality also other chlorides exist (cf. GUDIKSEN (1943))). Sodium is proved to be diffusible through the gastric mucosa by experiments with radioactive sodium — COPE, COHN & BRENIZER (1943).

TEORELL (1947) made a mathematical evaluation for the diffusion of alkali ions into the stomach contents. He considered the alkali ions as being mainly sodium ions. Denoting the steady state value to which the sodium concentration would approach in a resting stomach as S_0 and the permeability coefficient for sodium as r the following formulae were deduced (v , p and t retain their previous significance, cf. page 48):

$$\text{increment due to diffusion} = \frac{r(S_0 - Na) dt}{p + vt} \quad (59)$$

$$\text{decrement due to dilution} = - \frac{v dt}{p + vt} \cdot Na \quad (60)$$

The total concentration changes of sodium will then be

$$\frac{dNa}{dt} = \frac{S_0 r - (r + v) Na}{p + vt} \quad (61)$$

which gives

$$Na = \frac{S_0}{\frac{v}{r} + 1} \left[1 - \frac{1}{\left(1 + \frac{vt}{p} \right)^{\frac{r}{r} + 1}} \right] \quad (62)$$

If $p = 0$ the expression (62) is reduced to

$$Na = \frac{S_0}{\frac{v}{r} + 1} \quad (63)$$

which expresses a hyperbolic curve starting at S_0 for $v = 0$ and approaching the v -axis as its asymptote for increasing v -values.

If we know the hydrogen as well as the sodium ion concentrations we can calculate the total chloride content (acid chloride + neutral chloride). Thus

$$Cl = H + Na \quad (64)$$

or according to eqs (35) (page 48) and (63)

$$Cl = \frac{C_0}{\frac{k}{v} + 1} + \frac{S_0}{\frac{v}{r} + 1} \quad (65)$$

This eq. represents a curve which starts at S_0 for $v = 0$ and ends at C_0 for $v = \infty$ (cf. fig. 32). Between these two extremities the course of the curve depends on the interrelations between C_0 , S_0 , k and r .

The relation between chloride concentration and acidity can be calculated from (63) and (64)

$$Cl = H + \frac{S_0}{\frac{v}{r} + 1} \quad (66)$$

Eq. (35) (page 48) can be arranged to give

$$v = \frac{k}{\frac{C_0}{H} - 1} \quad (67)$$

Substituting (67) in (66)

$$Cl = \frac{(k - r)H^2 + r(C_0 - S_0)H + rC_0S_0}{(k - r)H + rC_0} \quad (68)$$

This relation is seen in fig. 33.

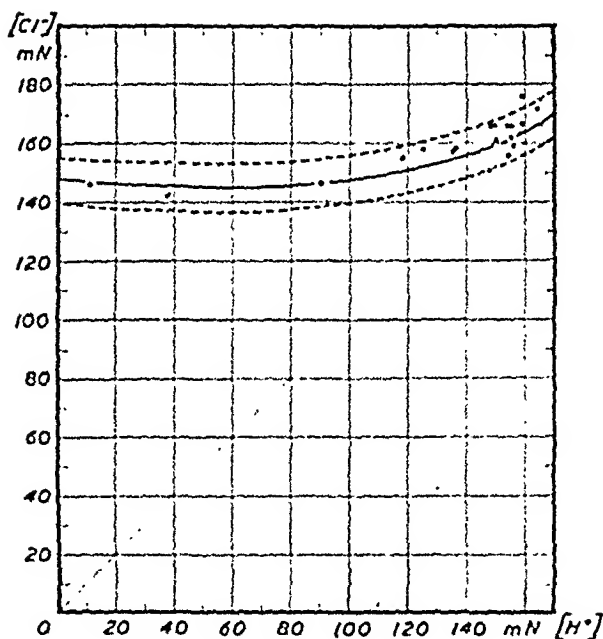


Fig. 33. The relation between the chloride concentration and the acidity. The experimental data are fitted with eq. (68) by the method of least squares. $S_0 = 151 \pm 7$ mN; $r = 0.06 \pm 0.01$ ml/min; $C_0 = 168$ mN; $k = 0.10$ ml/min. Standard error of estimate (the dotted lines) $= \pm 8$ mN.

Mathematical evaluation of the experimental data: By using the method of least squares S_0 and r can be determined in the eq. (68) if C_0 and k retain their previous values found in chapter H.

The statistical results are shown in the legend of fig. 33; the regression is represented by the full line and \pm the standard error of estimate by the dotted ones.

The best fit with eq. 65 was, however, found by trial and error. The regression is represented by the full line in fig. 32.

The chloride accumulation in the stomach.

The value of S_0 .

At first sight the results seem to give a simple explanation of the chloride concentration in the gastric acid, but there are some details that are rather questionable.

Both in the instillation experiments — TEORELL (1933), IHRE (1938) ELLIOT, RISHOLM & ÖBRINK (1942) — and in the present experiments, the steady state value for chloride in a non-secreting stomach is about 140—150 mN. It should be pointed out that the chloride content of

the blood (plasma) is only 100—110 mN (both in man and dog). On account of the Donnan effect a slight difference is to be expected.

It has been suggested — NIELSEN (1941)— that the ions in the plasma in man may have the following concentrations:

| Cations: | | Anions: | |
|------------------|---------|--------------------------------|-------|
| Mg ⁺⁺ | 2.5 mN | Protein | 20 mN |
| Ca ⁺⁺ | 5.0 » | Organ. acids | 2 » |
| K ⁺ | 5.1 » | SO ₄ ⁻⁻ | 1 » |
| Na ⁺ | 142.4 » | HPO ₄ ⁻⁻ | 3 » |
| | | HCO ₃ ⁻ | 28 » |
| | | Cl ⁻ | 101 » |
| Total 155 mN | | 155 mN | |

Assuming all these ions *except the proteins* (20 mN) to be diffusible through the gastric mucosa and denoting the total cations B (basic ions) and the total diffusible anions A (acid ions) the Donnan equilibrium states (cf. BLADERGROEN (1945), p. 325):

$$\frac{B_{\text{blood}}}{B_{\text{stomach}}} = \frac{A_{\text{stomach}}}{A_{\text{blood}}}$$

or

$$\frac{155}{x} = \frac{x}{135}$$

which makes $x = 145$ mN.

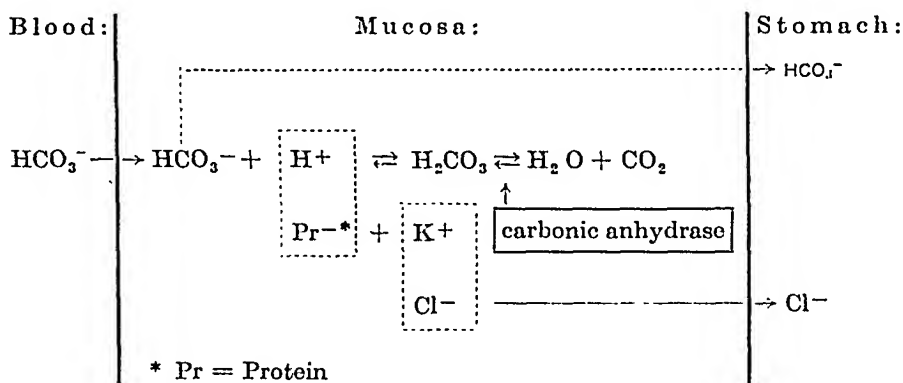
The diffusible anions would thus have a concentration difference between the gastric juice and the plasma of $145 - 135 = 10$ mN according to the Donnan distribution. The individual anions would then have a concentration in the gastric juice that is $\frac{145}{135}$ times higher than in the plasma. *For the chloride ions that would mean an increase from 100—110 to 107—118 mN and for the bicarbonate ions from 28 to 30 mN.* The bicarbonate ions would, however, be converted to carbon dioxide as soon as any acid secretion started in the stomach. It has, however, been impossible to obtain any considerable CO₂ production in the stomach — TEORELL (1933) — which perhaps may indicate that *the bicarbonate ions have never entered the stomach cavity.* The chlorides on the other hand have apparently accumulated in the stomach to a much higher concentration (ca 145 mN) than would be expected from the Donnan equilibrium formulae (107—118 mN). The following *theory* is proposed as an attempt to explain this phenomenon.

Theoretical.

DAVENPORT (1939) showed one part of the gastric mucosa to be very rich in *carbonic anhydrase*, which he thought to be essential for the acid production (for these theories cf. DAVENPORT (1939, 1940, 1941, 1942), DAVENPORT & FISHER (1940) and BULL & GRAY (1945)).

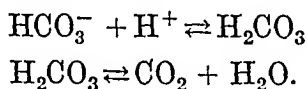
In a recent paper (1946) DAVENPORT himself, however, considers the carbonic anhydrase to be unimportant for the production of the hydrochloric acid.

It may, however, have a definite influence on the diffusion process of the bicarbonate ions according to the following schematical drawing:



If the mucosa is supposed to have the same concentration of protein as the blood plasma — 20 mN — (whether or not this proposition is correct will have no influence on the final result) the bicarbonate entering the mucosa will have the same concentration on both sides of the boundary “blood-mucosa.” This equilibrium will be valid also for the Cl^- ions.

When the bicarbonate ions arrive in the carbonic anhydrase “barrier” the following reactions may occur:



The CO_2 is probably very easily reabsorbed as was found by LINDE & TEORELL (1947) when they introduced NaHCO_3 into a secreting tied-off stomach.

At pH 6.48 (the “apparent” first dissociation constant = $10^{-6.48}$) 50 per cent of the total CO_2 is present as $\text{H}_2\text{CO}_3 + \text{CO}_2$. Owing to the disappearance of the gas CO_2 and to the catalytic action of carbonic anhydrase the decomposition of H_2CO_3 and the de-ionisation of HCO_3^- will proceed very easily.

The hydrogen ions used in the de-ionisation may be taken from the buffer systems in the mucosa, e. g. the proteins. These are presumably negatively charged at the pH in question.

In the schematical drawing the reaction between the hydrogen ion and the bicarbonate leaves the ions Protein^- , K^+ , and Cl^- . K^+ and Protein^- (which is indiffusible) interact and to preserve electrical neutrality, Cl^- has to leave the mucosa. *Thus the anion which entered the mucosa (HCO_3^-) has been substituted by the Cl^- ion.* This result is quite analogous to the *chloride shift in the red blood corpuscles*.

At the boundary "mucosa-stomach" the following situation may then be expected:

| | Mucosa: | | Stomach: |
|-----------------------|-------------------------|---------------|-------------------------|
| Plasma: | Cl^-
110 mN | \rightarrow | Cl^-
118 mN |
| Bicarbonate exchange: | Cl^-
28 mN | \rightarrow | Cl^-
30 mN |
| | | | 148 mN |

One can imagine the chloride ions as originating from two separate sources. *First* the 110 mN from the plasma which have penetrated the mucosa and *secondly* the 28 mN from the mucosa itself which have been substituted for the bicarbonate ions from the plasma.

These chloride concentrations will increase in the stomach according to the Donnan equilibrium. (The mucosa was supposed to have the same amount of indiffusible ions as the plasma — 20 mN —, and the stomach content is considered to be practically free from proteins). Thus the concentration of Cl^- in the stomach would be $118 + 30 = 148$ mN.

The net result may be summarized as the following:

| Blood: | | Mucosa: | | Stomach: |
|---------------------------|---------------|---------------------------|---------------|-------------------------|
| Cl^-
110 mN | \rightarrow | | \rightarrow | Cl^-
118 mN |
| HCO_3^-
28 mN | \rightarrow | HCO_3^-
28 mN | \rightarrow | Cl^-
30 mN |
| | | | | 148 mN |

The related process makes the gastric mucosa "apparently" impermeable to the bicarbonate ions. From a physical-chemical point of view this impermeability can only be "apparent" because a perfect impermeability to both the proteins (20 mN) and the bicarbonate ions (28 mN) would make only $155 - (20 + 28) = 107$ mN anions

permeable. This would give a concentration in the stomach of $\sqrt{155 \times 107} = 129$ mN total anions.

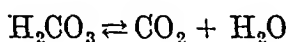
This is not in accordance with the experimental data (145 mN in the stomach), which suggest that only 20 mN are indiffusible (the proteins!). The bicarbonate ions do not give rise to any significant diffusion effects owing to slow diffusibility — TEORELL (1935 b, 1937 d) — or to total indiffusibility — DONNAN (1911).

As the bicarbonate ions have no diffusion effects and do not enter the stomach they may have been substituted by chloride ions as suggested above.

This theory has to some extent been supported by experimental data.

Experimental.

The essential part of the theory was the reaction



which was believed to depend on the presence of carbonic anhydrase.

As shown by DAVENPORT (1940) this catalytic action can be inhibited by *thiocyanate*. Doses of 50—200 mg NaSCN were therefore injected intravenously into narcotized cats, into the stomach of which HCl had been instilled. Fig. 34 shows such an experiment. The stomach was tied off and 5 ml 170 mN HCl were introduced and examined every 15 minutes until the chloride concentration had reached a steady state level (about 145 mN). Then 200 mg thiocyanate were injected. In about 30 minutes the chloride concentration tended to decrease and to approach the chloride concentration in the plasma (109 mN). At the same time the samples began to foam. The foaming was shown not to depend on any increment of proteins in the gastric contents.

This is in accordance with the theory. When the carbonic anhydrase was inhibited the mucosa might have become permeable towards the bicarbonate ions (as indicated by the dotted arrow in the first schematic drawing) which entered the stomach yielding CO_2 on reacting with the instilled HCl. As a result no chloride would substitute the bicarbonate in the stomach, and the chloride concentration would approach the values indicated by the Donnan equilibrium (117 mN).

In order to prove that the changes in the chloride concentration

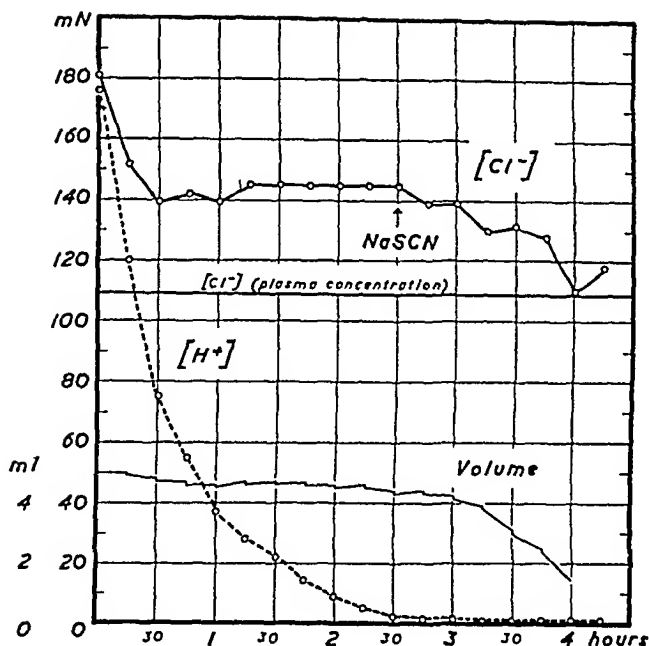


Fig. 34. Influence of NaSCN on the chloride accumulation in the stomach. An instillation of 170 mN HCl in a cat's stomach. When a steady state level of the chloride concentration had been reached NaSCN (200 mg) was injected intravenously.

really were accompanied by changes in the diffusibility of the bicarbonate ions, experiments of the following nature were performed (Table 9):

Table 9. Instillation of 0.20 M glycol in a stomach of a cat. A new 5.0 ml volume was introduced every period. (* denotes that 200 mg NaSCN were injected intravenously at the beginning of a period.)

| Time
Minutes | Volume ml | | pH | Total
acidity
mN | Total
chloride
mM | Bicar-
bonate
mN |
|-----------------|-----------|-----------|-----|------------------------|-------------------------|------------------------|
| | instilled | recovered | | | | |
| 0—15 | 5.0 | 5.0 | 4.2 | 11 | 39 | 3.9 |
| 15—30 | 5.0 | 5.0 | 4.4 | 7 | 33 | 0.5 |
| 30—45 | 5.0 | 5.0 | 4.2 | 8 | 30 | 3.0 |
| 45—60 | 5.0 | 5.0 | 4.3 | 8 | 33 | 4.4 |
| 60—90 | 5.0 | 5.1 | 4.0 | 14 | 37 | 0.5 |
| 90—105* | 5.0 | 4.7 | 4.3 | 8 | 33 | 6.7 |
| 105—120 | 5.0 | 4.9 | 6.7 | 3 | 19 | 7.1 |
| 120—135* | 5.0 | 5.1 | 7.4 | — | 21 | 6.9 |
| 135—150 | 5.0 | 5.2 | 7.5 | — | 19 | 6.5 |
| 150—180* | 5.0 | 5.1 | 7.7 | — | 25 | 11.7 |
| 180—195* | 5.0 | 4.7 | 7.7 | — | 25 | 8.9 |

0.20 M glycol solution (which does not cause any stimulation or inhibition of the secretion and which does not change its volume in a non-secreting cat's stomach when introduced into it) was kept in the stomach of a cat for 15 or 30 minutes. After this time the bicarbonate content was determined (for method see page 18). The same procedure was then repeated in the same cat after injection of thiocyanate. It was found that the concentration of bicarbonate showed a marked increase after the thiocyanate administration. At the same time the chloride concentration appears to have decreased. The differences in acidity may depend on neutralization by bicarbonate after the thiocyanate administration.

This seems to be further evidence of the correctness of the first part of the theory. The rest of it has, unfortunately, no experimental support beyond the behaviour of the chlorides.

CHAPTER 13.

Total Output of Chloride in Relation to the Secretion Rate.

Theoretical considerations.

According to formula (65) (page 74)

$$Cl = \frac{C_0}{\frac{k}{v} + 1} + \frac{S_0}{\frac{v}{r} + 1}$$

The total amount of Cl will then be

$$vCl = \frac{vC_0}{\frac{k}{v} + 1} + \frac{vS_0}{\frac{v}{r} + 1} \quad (69)$$

which can be transformed to

$$vCl = vC_0 - k \frac{C_0}{\frac{k}{v} + 1} + rS_0 - r \frac{S_0}{\frac{v}{r} + 1} \quad (70)$$

and by substituting (35 and 63) (page 48 and 74)

$$vCl = vC_0 - [kH - r(S_0 - Na)] \quad (71)$$

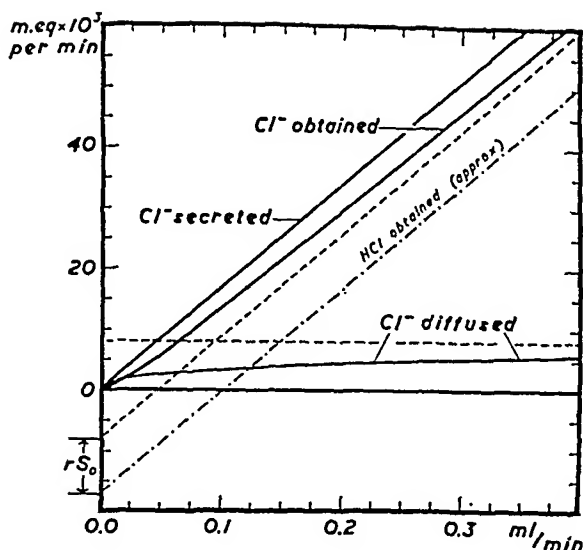


Fig. 35. *The theoretical behaviour of the chloride ions in the stomach.* The Cl^- obtained approaches the interrupted oblique line as its asymptote. rS_0 denotes the neutral chlorides.

The expression vC_0 is the amount of chloride secreted as hydrochloric acid, $r(S_0 - Na)$ is the amount that has diffused into the stomach as neutral chloride and kH the amount which has left the stomach by the diffusion of HCl .

The difference $[kH - r(S_0 - Na)]$ is the net result of the chlorides that have diffused out of the stomach — see fig. 35: “ Cl^- diffused.” In this figure vC_0 is “ Cl^- secreted” and the vCl is “ Cl^- obtained.” The line “ HCl obtained (approx)” is the same as in fig. 30 (page 69).

If $\frac{k}{v}$ and $\frac{r}{v} \ll 1$ the eq. (69) can be changed to

$$vCl = vC_0 \left(1 - \frac{k}{v}\right) + rS_0 \left(1 - \frac{r}{v}\right) \quad (72)$$

and if $r \leq 1$

$$vCl = vC_0 - (kC_0 - rS_0) \quad (73)$$

This approximated formula differs less from (71) than did the corresponding acidity curve (58) from the formula calculated for it (56). Cf. page 68.

As vCl and v are the only variables in (73) this equation is expressed by a straight line, the slope of which is the same as for (58) ($\text{tg } \alpha = C_0$). The vCl -intersection is $-(kC_0 - rS_0)$ and the distance between the approximated Cl^- and HCl curves in fig. 35 is rS_0 or the total amount *neutral chloride*.

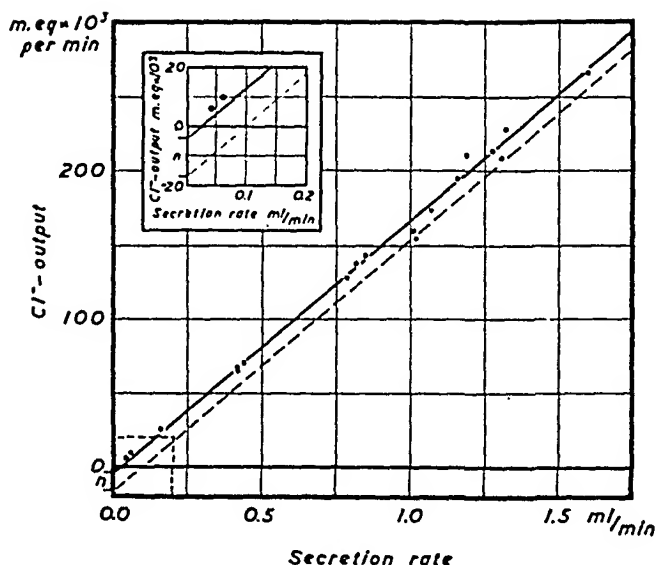


Fig. 36. Relation between the amount of chloride obtained and the secretion rate. The dotted square in the lower left-hand corner is enlarged in the inset. The oblique dotted line is the amount HCl obtained (cf. fig. 31) and n denotes the neutral chlorides (cf. fig. 35).

Experimental data.

The relation between the total chloride output and the secretion rate is seen in *fig. 36* (dog B). The experimental results are smoothed with a straight line (freehand) corresponding to the eq. (73).

In the figure the approximated HCl curve is also seen. Its distance from the chloride curve is denoted by n (neutral chloride).

The points do not agree as well as the corresponding acidity values with the theoretical curve, but are sufficiently well fitted by the line to suggest that the theory may be correct.

The chloride curve is analogous to that given by GRAY, BUCHER & HARMAN (1941). They tested their curve statistically and found that it had a somewhat greater angle of inclination than the HCl curve. From this it follows that the amount of neutral chlorides would increase with increasing secretion rates and that the concentration of neutral chloride would never approach zero for infinitely large secretion rates.

The difference between their chloride curve and the author's is probably not significant and may be a matter of experimental errors, as can be seen from their statistical values. The slopes of their chloride and acidity curves do not differ statistically. The present curves are parallel owing to the physical-chemical postulates.

CHAPTER 14.

The Excretory Function of the Parietal Cells. Neutral Red Elimination.

Introduction.

Since FULD (1908) discovered that *neutral red* (N. R.), when poured into the main stomach of a Pavlov dog, appeared in the pouch, many investigations concerning the elimination of this dye (and many others) have appeared. — For a good review of the earlier literature see KOLM, KOMAROW & SHAY (1945). In several clinical studies a *qualitative* relationship between the secretory function of HCl and the elimination capacity of N. R. has been found. In some cases of achylia the dye did not appear at all and in cases of hyperacidity the “appearance time” after an intramuscular or intravenous injection was shorter than normal.

It was shown by HAMPERL — see GLAESSNER & WITTGENSTEIN (1925) — that an accumulation occurred in the parietal cells. Several subsequent experiments have made it appear probable that the dye is exclusively secreted by the parietal cells — KATSCH & KALK (1926), TEJIMA (1935), MORRISON (1938), KOLM, KOMAROW & SHAY (1945). Papers have appeared, however, in which a contrary opinion is maintained as to the place of the excretion. INGRAHAM and VISSCHER (1935) have shown that all the dyes eliminated by the gastric mucosa are cationic, i. e. they have a basic chromophoric group.

Besides the stomach, the kidneys and the liver participate in the elimination of neutral red.

In a forthcoming paper by HALLÉN (1948) some experiments are described which show that in total achylia (pernicious anaemia) the N. R. can be extracted by instillation into the stomach of 150—170 mN hydrochloric acid though it is not excreted after histamine stimulation. HALLÉN has also been able to show that N. R., when instilled into the stomach, is reabsorbed by the stomach walls.

Quantitative estimations of the N. R. elimination are rare and give no definite relationship between the N. R. concentration of the gastric juice and the secretion rate of the acidity, though it has been suggested that the concentration of N. R. in the juice runs parallel with the secretion of hydrochloric acid — KOBAYASHI (1926), MARUNO

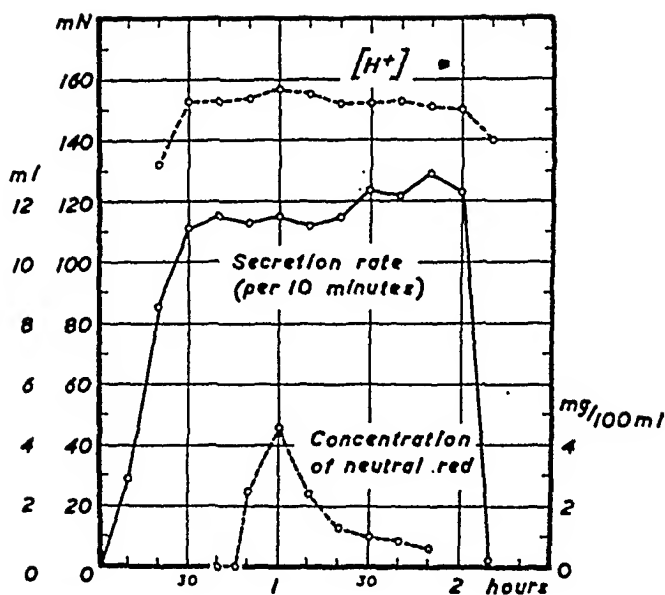


Fig. 37. The effect of an intramuscular injection of neutral red. The secretion rate was induced by histamine. At 0th hrs the N.R. was injected.

(1935) and MORRISON (1938). Also cf. KOLM, KOMAROW & SHAY (1945).

It was therefore thought most desirable to examine the quantitative relationship between the excretory and the secretory function of the stomach and therefore experiments with neutral red were performed with an improved technique.

Experimental.

The same dogs as in the previous chapters were used (dogs R, S and B). In every experiment the secretion was induced by the continuous intravenous injection of histamine (cf. page 10).

The neutral red was dissolved in water, 1 gram per 100 ml, filtered and sterilized. The final strength of this solution was 0.940 per cent.

In a preliminary experiment the dye was injected intramuscularly with the result given in fig. 37. After 6 minutes it appeared in the gastric juice and the concentration rose very quickly to a maximal value within 20 minutes. Then it decreased again. (A slight secretagogue effect could be noticed). It is quite easy to understand that attempts to find the possible interrelation between the secretion and the excretion in this way are extremely difficult. It was therefore necessary to have a constant concentration of neutral red in the blood

during the observations. This could be obtained by injecting the dye intravenously at a constant continuous rate.

The method finally adopted was the following: the injection of the dye was started when a constant secretion rate had been obtained by histamine. It was found necessary to continue the injection of neutral red for at least three hours in order to get a constant colour of the juice. Only the corresponding *steady state values* were used when computing the relationship between the secretion rate and the excretion power.

For the methods of analyzing the concentration of the neutral red in the gastric juice see page 19.

Results.

Constant continuous intravenous injections of N. R. with varying secretion rates.

With the technique described the concentration of neutral red was held constant by the continuous intravenous injection during many experiments, but the injection rate of histamine was varied, thus causing varying secretion rates. A typical experiment of this kind is seen in *fig. 38*.

The result obtained by plotting the steady state values from several similar experiments in a coordinate system is shown in *fig. 39* (dog B).

The concentration of N. R. in the juice was higher the lower the secretion rate. The same result was obtained in all the dogs used.

The *total amount* of N. R. eliminated through the pouch was calculated by multiplying the concentration by the volume secreted and gave the relation to the secretion rate as shown in *fig. 40*. This seems to be an almost linear relationship, so that *the total amount eliminated by the gastric juice increased with increasing secretion rates.*

It might be of interest to compare the amount injected with the amount eliminated by the stomach.

In the experiments described 37.6 mg/hour have been injected and at the highest secretion rate (1.42 ml/min) 16.67 μ g/min were eliminated (cf. *fig. 40*). This makes $\frac{16.67 \times 60}{1000} \approx 1$ mg/hour eliminated.

Considering the pouch to be about 1/4 of the entire stomach, 4 mg neutral red were eliminated per hour. That makes about 10 % of the amount injected. It ought to be remembered that the computations

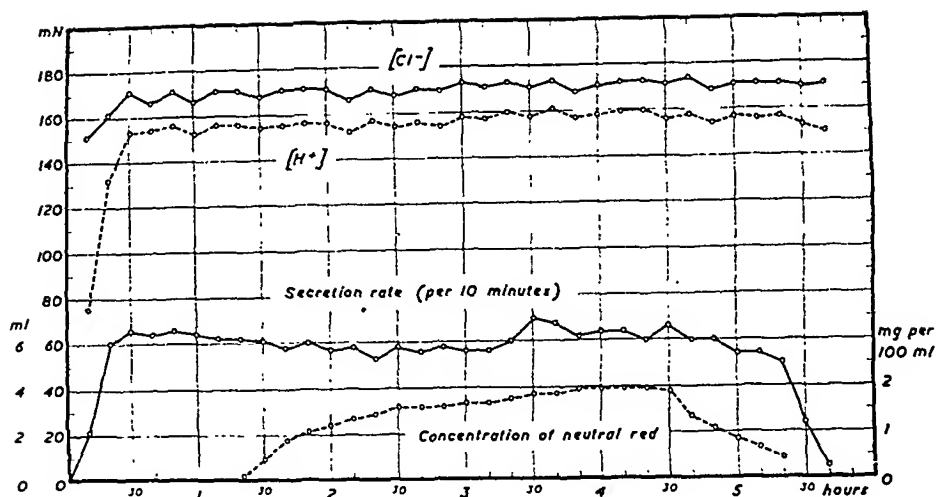


Fig. 38. A typical experiment with continuous intravenous injection of histamine and neutral red. When the secretion rate was constant the injection of N.R. was started. The injection of N.R. was stopped at 4³⁰ hrs and that of histamine at 5²⁰ hrs.

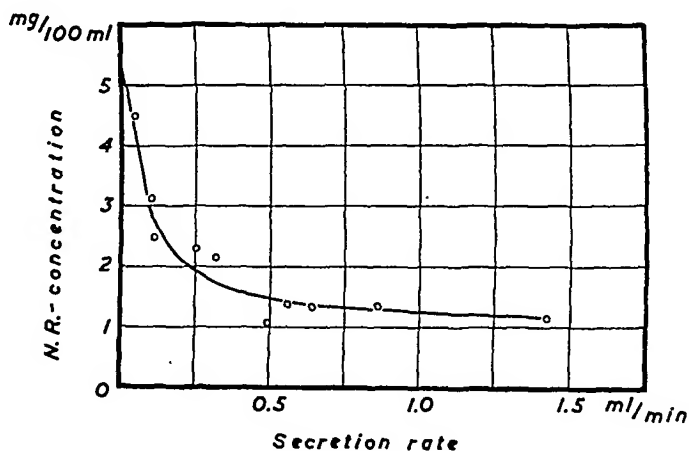


Fig. 39. The relation between the concentration of N.R. in the gastric juice and the secretion rate. N.R. was injected at a constant rate (37.6 mg/hour). Every value represents a steady state level.

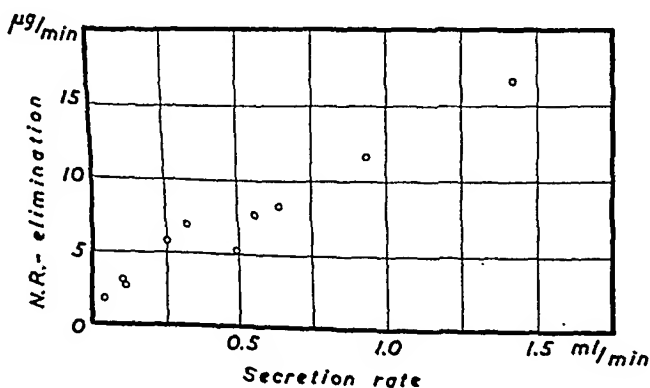


Fig. 40. The relation between the total amount of N.R. eliminated by the gastric juice and the secretion rate. The values are obtained from fig. 39.

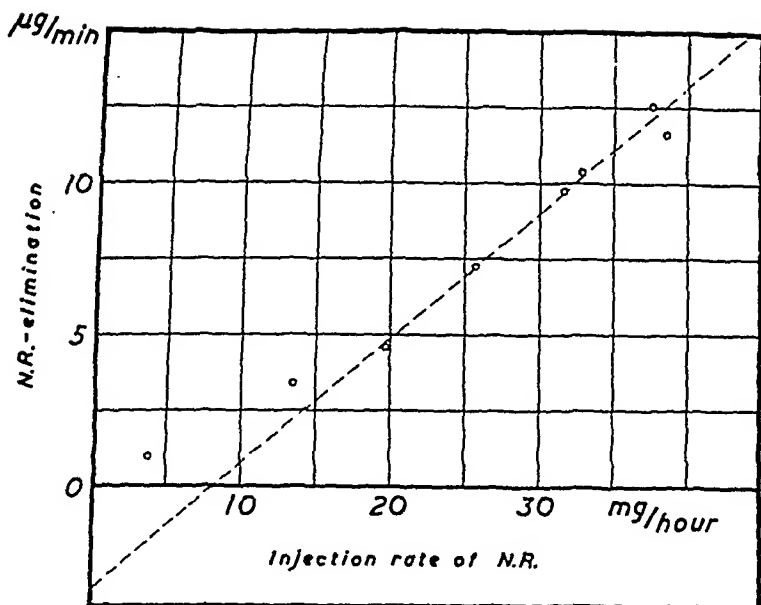


Fig. 41. The relation between the injection of N.R. and the amount eliminated by the gastric juice. The secretion rate was kept constant (cf. Table 10). Every value is obtained from a steady state level. The freehand dotted line gives an intercept on the ordinate which may indicate a resorption of N.R. from the gastric pouch.

were made during steady state conditions, i. e. the amount eliminated equals the amount injected. (As 37.6 mg/hour were injected the same amount is considered to be eliminated.) Thus *the stomach is responsible for at most about 10 % of the total elimination of neutral red.*

This fact may be discussed as it invalidates to some extent the suggestion made above that the injection rate of neutral red is directly proportional to the steady state value of the concentration in the blood. With an increased secretion rate the elimination was increased (fig. 40). The theoretical basis for a straight line relation between the injection rate and the steady state concentration was, however, the constancy of all diffusion and elimination constants (cf. chapter 4 page 20).

It is considered, however, that the changes in the elimination will only have a slight influence on the concentration in the blood.

Influence of the blood concentration of neutral red upon the elimination by the gastric mucosa.

As is seen from the foregoing, the secretion rate has a definite influence on the elimination capacity of N. R. by the gastric mucosa. In order to investigate the relation between the blood concentration of N. R. and the elimination it is therefore essential to have a constant

Table 10. *The influence of different injection rates of neutral red on the elimination of the dye in the gastric juice. All values are obtained from steady state levels.*

| Injection rate of N: R. mg/hour | Secretion rate of gastric juice ml/min | Acidity mN | NR in the gastric juice | |
|---------------------------------|--|------------|-------------------------|--------------------|
| | | | Concentration mg/100 ml | Amount μ g/min |
| 3.9 | 0.57 | 135 | 0.18 | 1.02 |
| 13.5 | 0.63 | 146 | 0.54 | 3.38 |
| 19.7 | 0.67 | 153 | 0.69 | 4.59 |
| 25.7 | 0.60 | 151 | 1.22 | 7.32 |
| 31.6 | 0.66 | 153 | 1.48 | 9.77 |
| 32.9 | 0.55 | 153 | 1.90 | 10.36 |
| 37.6 | 0.67 | 168 | 1.80 | 12.06 |
| 38.5 | 0.60 | 159 | 1.95 | 11.70 |

and identical secretory rate in all experiments. If a large secretion rate is chosen small changes will, however, have but a slight influence as seen from fig. 39.

This was done in dog R and in these experiments the secretion rates varied between 0.55—0.67 ml/min.

The injection rates of N. R. can be considered as proportional to the steady state values of the blood concentration.

The results are shown in *Table 10* and *fig. 41*, where the total amount (μ g/min) eliminated by the pouch is plotted against the injection rate of N. R. (= the blood concentration of N. R.).

The points lie on a curve which, at least for the higher values of injection rates, seems to be a straight line. This is shown as the dotted line in the figure.

It is quite clear from this curve that *the rate of elimination will be dependant on the concentration of the dye in the blood*. This is the reason why the curve in fig. 37 has its particular shape after an intramuscular injection of N. R., as the blood concentration of N. R. probably has a similar time-concentration relation.

Attempts have been made to analyze the concentration of N: R. in the blood but, even during the largest injection rates of N. R., no N. R. could be identified in the blood. Acidifying, oxidation with ceric sulphate in an acid solution, extraction with ether-alcohol mixtures gave all the same negative results. This indicated that an enormous accumulation had occurred in the gastric mucosa, as described by KOLM, KOMAROW & SHAY (1945) and others.

Discussion.

With the aid of the continuous intravenous injection technique, both the concentration of neutral red in the blood and the secretion rate of gastric acid were held at constant steady state levels. This made it possible to study the relation between the secretion rate and the elimination of the dye in the stomach as well as the influence of the blood concentration on this elimination.

The interesting relationship between the concentration of N. R. in the gastric juice and the secretion rate may perhaps be explained by different mechanisms.

A probable one may be that N. R. is accumulated to a high concentration in the mucosa cells (the parietal cells) while the *intercellular* fluid also contains some dye but at a significantly lower concentration. Such a difference in the concentration between cells and surroundings is shown to exist in an aqueous solution of baker's yeast — COLLANDER and ÄYRÄPÄÄ (1947). The water for the hydrochloric acid secretion is supposed to be taken from the intercellular fluid thus giving a gastric secretion which is mixed up with the dye from the beginning. While passing through the intra- and/or intercellular canaliculae the secretion (now with a low pH) will take up more N. R. from the cells by a diffusion process. This diffusion may be more significant at small secretion rates. Thus the relation between the secretion rate and the concentration of N. R. in the juice will be that found experimentally.

The processes can be described by the regression line

$$C = C_0 - (C_0 - C_\infty) e^{-\frac{k}{r}} \quad (74)$$

which is shown in fig. 39. Here C is the concentration of N. R. in the juice; r the secretion rate; the constants C_0 and C_∞ the concentration in the juice at infinitely small and high secretion rates; k a constant.

This regression line is quite well fitted by the experimental data as seen in fig. 39. Whether or not the theoretical basis is correct the equation is a good expression of the relationship between the secretion rate and the concentration of neutral red in the gastric juice.

It may therefore be justifiable to use this equation in fig. 40 as well. The total amount secreted will then be

$$rC = rC_0 - r(C_0 - C_\infty) e^{-\frac{k}{r}} \quad (75)$$

From chapter 10 the relation between the secondary acidity and the secretion rate is known to have a shape quite different from the N. R. concentration just described. That means that *there is no parallelism between the secondary acidity and the concentration of neutral red.*

One should keep in mind the relationship between the secretion rate and the primary acidity (fig. 21, page 45). The resemblance between the two curves may or may not be determined by a common factor.

The elimination depends not only on the secretion rate, but also on the concentration of the dye in the blood and tissue (fig. 41). It can be deduced from the theoretical basis given above, and it is quite possible and plausible, that the amount eliminated may be directly proportional to the blood and tissue concentration of the dye. This would give a straight line relation passing through the origin.

Any deviation from such an expected curve that may be shown by the experimental data in fig. 41 might be a result of back diffusion of neutral red through the gastric mucosa. This would be in accordance with the results obtained by HALLÉN (1948).

The practical consequences of the above may be that the clinical use of neutral red is limited to *qualitative* experiments based on appearance or absence or on an "appearance time" of the dye, unless a laborious method like the present one is used.

Conclusions: The elimination of neutral red was found to be dependant on both the secretion rate and the concentration of the dye in the blood. The secondary acidity does not run parallel with the concentration of neutral red in the gastric juice.

Intramuscular (or massive intravenous) injections do not afford any possibility of investigating the correlations between the elimination and any properties of the parietal secretion.

The eliminated dye is supposed to come both from the intercellular fluid in the course of the process of secretion of HCl and from the cells — which are supposed to accumulate the dye — by a diffusion process.

CHAPTER 15.

Influence of Enterogastrone on the Parietal Secretion.**Historical.**

Since more than fifty years many authors have reported that fat given to an animal depressed the secretion of gastric acid — EWALD & BOAS (1886), CHISCHIN (1894), WIRSCHUBSKI (1900), FERMI (1901), WALKO (1903), KASANKI (1903). Fat was also used against hyperacidity and ulcers — COHNHEIM (1904), EBSTEIN (1904).

SZOKOLOW (1904) separating the duodenum from the stomach in a dog, found that it was only the presence of fat in the duodenum which suppressed the secretion of HCl in the stomach. The effect was not abolished by vagotomy — LIM, IVY & MCCARTHY (1925) — and was found even in a completely denervated gastric pouch — FENG, HOU & LIM (1929).

On the basis of these experiments it has been suggested that the presence of fat in the duodenum causes it to secrete an active principle which has the effect of diminishing gastric secretion. KOSAKA & LIM (1930) suggested the name *enterogastrone* for this active principle, which could be extracted from the mucosa of the small intestine.

Important papers concerning enterogastrone have appeared by IVY and his co-workers. GRAY, BRADLEY & IVY (1937) developed a method of assay for a purified enterogastrone preparation. HANDS, GREENGARD, PRESTON, FAULEY & IVY (1942) discovered that *enterogastrone prevented experimental jejunal ulcers* in dogs. This stimulated further work in this field — HANDS, GREENGARD, FAULEY & IVY (1943), GROSSMAN, GREENGARD, DUTTON & WOOLLEY (1944), IVY (1944), GROSSMAN & IVY (1946). Also cf. the paper about pepsin secretion and enterogastrone by GROSSMAN, GREENGARD, WOOLLEY & IVY (1944).

Enterogastrone preparations have also been used in the treatment of ulcers in man with good results by GREENGARD, ATKINSON, GROSSMAN & IVY (1946).

The chemical properties of purified enterogastrone.

ÖBRINK (1947 a) found that the active principle in *enterogastrone preparations* has no or at least a very slight electrophoretic mobility between

pH 2.06 and 7.42. WINBERG (1947) reported that the active principle in the same preparation was *easy to dialyse* through a cellophane membrane. He made use of this property and purified the preparation by dialysis. In the dialysate a very active concentrate was recovered.

This dialyzed enterogastrone could not be inactivated by the action of pepsin or trypsin — WINBERG & ÖBRINK (1948) — which led to the conclusion that it is *probably not a protein*. However, the nitrogen content is 15 %, which makes these results unexpected or may indicate that the preparation is still very impure. Nevertheless its biological activity is extremely high.

The physiological properties of enterogastrone.

Several experiments have been performed on the dogs using this enterogastrone preparation (Eg)¹. In this paper the main question is in what sense it has any influence on the parietal secretion. Besides this there is, however, one interesting thing which ought to be reported here in a preliminary way.

In most experiments the Eg was injected intravenously in a massive dose. 50 mg were enough to give the following symptoms:

If the injection is made too rapidly (c. g. in about 30 seconds) the dogs become *unconscious* and develop *convulsions*. This attack starts in about one minute after the beginning of the injection and lasts for about five minutes. Afterwards the dog is rather tired. During the attack the bladder is emptied and defaecation sometimes occurs.

If, at the start of the attack or during it, some mls of a 5 % glucose solution is injected intravenously the dog's recovery is immediate and complete.

There are many experiments which will have been carried out in this field before any conclusions can be drawn, but the phenomenon seems sufficiently interesting to deserve this preliminary report.

The influence of Eg on the parietal secretion.

In chapter 6 it was shown that there was a close relation between stimulus and secretion. When an inhibitory (or a stimulating) substance is to be tested on the dog with a pouch it is essential to stimulate the mucosa submaximally. This was pointed out by

¹ The preparations have been made by Dr WINBERG, A. B. Astra, Södertälje, Sweden.

ÖBRINK (1946), and is easily understood from fig. 14 (page 33). When the secretion rate is maximal, a stimulating agent may have no influence and the same may be true for inhibiting substances. It is therefore necessary to have a secretion which corresponds to the ascending part of fig. 14. In the present enterogastrone experiments this point has been under complete control.

As enterogastrone may be a substance which acts in the physiological regulation of the parietal secretion, a thorough investigation of its rôle seems justifiable.

The questions arising are:

What is the influence of enterogastrone on

1. the *histamine concentration* in the plasma,
2. the *secretion rate* in histamine-induced gastric secretion,
3. the *acidity* of this secretion,
4. the *chloride concentration* in it,
5. the *permeability properties* of the gastric mucosa and
6. the *neutral red excretion* by the parietal cells?

1. *Influence on histamine concentration in the plasma.*

From chapter 4 the relations between the injection rate of histamine and the plasma concentration are known. In chapter 6 page 30 this knowledge is used to obtain the relation between the concentration of histamine in plasma and the secretion rate.

By using Eg we have the possibility of depressing the gastric secretion, and the question now is whether or not this depression is dependant on a decrease in the concentration of histamine (histaminase effect).

In Table 11 this question is answered. When the histamine injection had resulted in a constant secretion rate, 50 mg Eg-preparation were given intravenously in about 15 seconds. (The attack described above occurred.) The secretion decreased and disappeared in spite of the continued histamine stimulation. The histamine analyses in plasma samples before and after the Eg injection showed no significant difference. (Because of shortness of material only one experiment could be made. Also cf. the error of the method of analyzing for histamine, page 16).

It may be stated that enterogastrone probably does not inhibit the gastric secretion by destroying the histamine in the blood.

Table 11. *The effect of enterogastrone on the secretion rate and the plasma concentration of histamine.*

| Injection
rate of histamine
mg/hour | Secretion
rate
ml/min | Histamine in
plasma
$\mu\text{g}/100\text{ ml}$ |
|---|-----------------------------|---|
| Before enterogastrone injection | | |
| 1.8 | 0.62 | 9.9 |
| After enterogastrone injection | | |
| 1.8 | 0.03 | 9.0 |

2. Influence on the histamine-induced gastric secretion rate.

As seen above the injection of Eg results in a decrease in the gastric secretion rate. This is also seen from *fig. 42* and *44*.

The present author has never observed a refractory state of the dogs towards enterogastrone such as was reported by GRAY & WIECZOROWSKI (1939) and by FRIEDMAN & SANDWEISS (1946) when they tested the gastric depressant in urine, *urogastrone*.

The effect has appeared at different times after the injection from a few minutes to 45 minutes. The longest time lag (45 minutes) was obtained with the most active preparations obtained from dialysates, but the significance of the time lag is quite unknown.

The effect has in many cases amounted to an absolute abolition of the secretion (as seen in *fig. 42*), and the effect has lasted from 3—7 hours.

In forthcoming experiments the correlation between the inhibitory effect and the dose of enterogastrone will be studied.

3. Influence on the acidity of the gastric secretion.

In *fig. 42* a typical experiment with an effective enterogastrone preparation shows the behaviour of the acidity.

When the secretion rate decreases the acidity follows but shows a considerable time lag. This time lag may, however, depend on the impossibility of emptying the pouch completely, and the resulting formation of a "lake" around the rubber tube (*fig. 27*) which was described in chapter 10 page 63.

A "lake" containing 4 ml such as has been found in the experiments

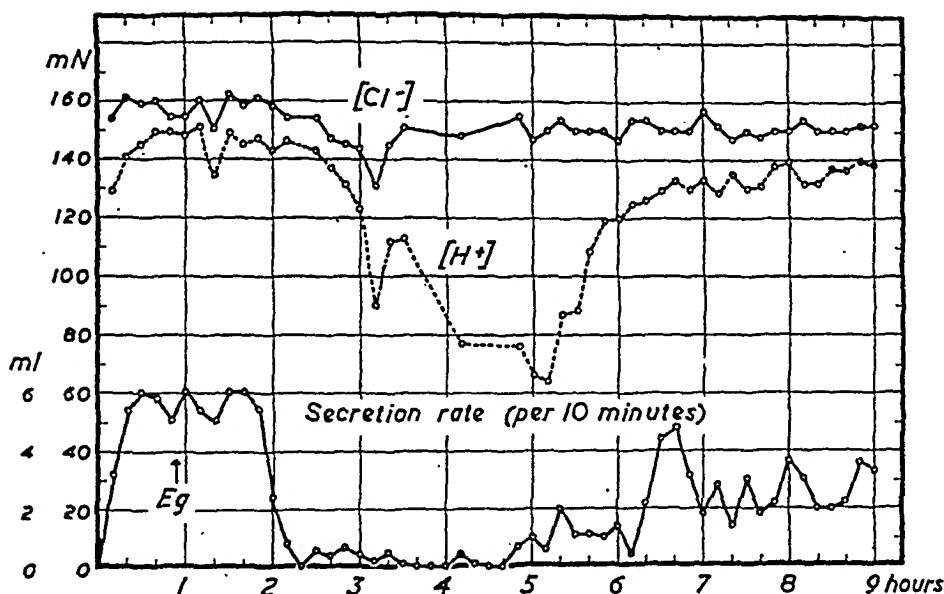


Fig. 42. The effect of enterogastrone (Eg) on the gastric secretion.

described on page 63, would explain the behaviour of the acidity curve.

It is most likely that all changes in the acidity are due to changes in the secretion rate, and that *there is no specific action by enterogastrone on the acidity.*

4. Influence on the chloride concentration of the gastric secretion.

As we know the relation between the chloride concentration and the secretion rate, we do not expect to find any considerable changes in the chlorides after the administration of Eg, provided that this has no specific action on the kinetics of the chloride secretion.

Fig. 42 shows that no such action is obtained. Therefore *enterogastrone has no direct influence on the chloride concentration in the gastric juice.*

5. Influence on the permeability properties of the gastric mucosa.

The decrease in acidity following the instillation of HCl has already been described (page 66). This reduction in acidity is supposed to be caused by an interchange of sodium and hydrogen ions across a diffusion membrane, chloride ions being the main anions on both sides of this membrane. It is of interest in the present connection to see whether this process is affected by enterogastrone.

Such instillation experiments have been performed on cats. Fig. 43

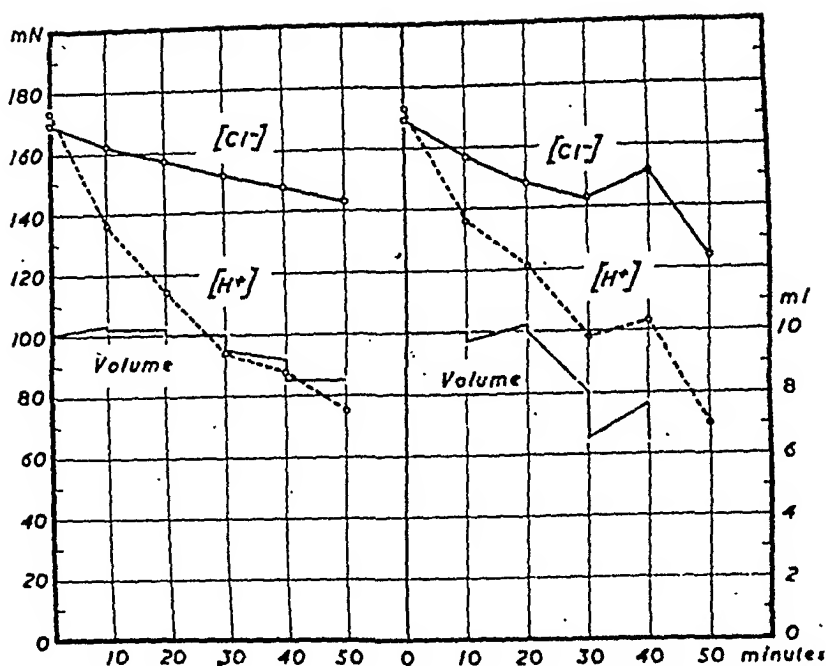


Fig. 43. The acidity reduction of an instilled volume HCl before and after administration of enterogastrone.

shows that the course of the curves is in agreement with normal instillation experiments, indicating that *enterogastrone seems to have no influence on the permeability of the gastric mucosa toward hydrochloric acid.*

6. Influence on the neutral red excretion by the gastric mucosa.

In chapter 14 page 84 ff. the relation between the excretion of neutral red (N. R.) and the secretion rate was considered. If the concentration of the dye was held at a constant level in the blood plasma by a continuous intravenous injection, the excretion of the dye was dependent on the rate of secretion as shown by fig. 39, page 87. The lower the secretion rate the higher the concentration of neutral red in the gastric juice.

In fig. 44 the influence of enterogastrone on this special property of the parietal cells is considered. The histamine injection is started at 0 hours. When the secretion rate has become constant the injection of neutral red is begun (2⁵⁶ hrs). When the colour of the secreted juice seems to be constant, enterogastrone is administered intravenously (4²⁰ hrs) and secretory inhibition results. The injection of N. R. is stopped at 5³⁶ hrs and that of histamine at 6²⁰ hrs.

After the administration of enterogastrone the secretory rate decreases to about 60 %. The N. R. concentration increases from 4

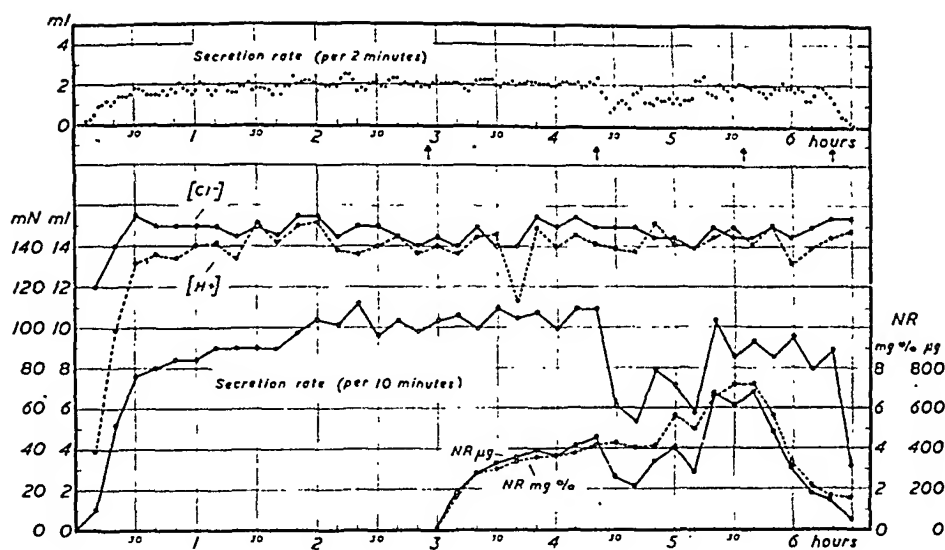


Fig. 44. The influence of enterogastrone on neutral red excretion. At 2⁵⁶ injection of N.R. is started, at 4²⁰ Eg is injected, at 5³⁶ and 6²⁰ the injections of neutral red and histamine respectively are ended.

to 7 mg/100 ml after a time lag which may be explained by the "lake" (page 63). This increase in the dye concentration is, however, expected from fig. 39 page 87.

It is therefore most probable that *enterogastrone* has no specific influence on the excretory function of the parietal cells.

Conclusions: — The above observations have shown that *enterogastrone* only affects the volume of secretion produced by the parietal cells. This effect is probably not due to any histaminase effect of *enterogastrone*.

No specific action on the acidity, chloride concentration or excretory function could be found after *enterogastrone* administration, nor did the permeability of the mucosa show any changes in respect to hydrochloric acid.

General Summary.

The Purpose of the Present Investigation.

The intention was to study the parietal cell secretion in the stomach from a kinetic point of view. CHAPTER 1 (page 7).

Procedure.

The investigations were carried out on Heidenhain dogs and, in some experiments, on cats.

Histamine was used as a stimulating agent and was given by continuous intravenous injection using a specially constructed injection apparatus which is described.

An improved technique for collecting the gastric juice was used. CHAPTER 2 (page 8).

Methods of Analysis.

All the methods employed for the analyses are described. They include the determinations of pH, acidity, chloride, pepsin, nitrogen, reducing power, calcium, bicarbonate, phenol red and neutral red in the gastric contents and of histamine in the plasma. CHAPTER 3 (page 14).

The Histamine Distribution in the Body.

The kinetics of the distribution of a drug administered to the body by means of a subcutaneous or continuous intravenous injection were discussed on the basis of theoretical considerations.

Experiments showed that — in agreement with the theoretical considerations — the histamine concentration in plasma was proportional to the injection rate of histamine. CHAPTER 4 (page 19).

Time-secretion Relation after Continuous Intravenous Injection of Histamine.

The secretion could be held at a constant rate by the stimulation technique employed. Any changes in the injection rate were followed by similar changes in the secretion rate. Under special conditions an *apparent* independence between stimulus and secretion could be obtained. CHAPTER 5 (page 26)

Relation between Histamine Concentration and Rate of Secretion. Concentration-action Curves.

It was suggested that the relation between the injection rate of histamine (r) and the secretion rate (v) could be described by the expression

$$v = a(1 - e^{-kr}),$$

where a = the maximal secretion rate; k = constant.

The concentration-action curve was treated according to the method given by GADDUM. CHAPTER 6 (page 30).

The Secretion of Gastric Juice after Subcutaneous Injections of Histamine.

The relation between the dose of histamine and the time-secretion curve was discussed. A close relation to the maximal secretion rate or to the total amount secreted was found only when small doses of histamine were used.

The, "continuous histamine test," i. e. frequently repeated subcutaneous injections of histamine, was discussed. CHAPTER 7 (page 37).

The Minimal Effective Dose of Histamine Producing Gastric Acid.

It was suggested that there exists no threshold value for the action of histamine in stimulating the gastric secretion. CHAPTER 8 (page 41).

On the Primary Acidity.

Reference was made to a recent investigation which suggested the possibility that the primary acidity increased with decreasing secretory rates. CHAPTER 9 (page 43).

Regulation of the Acidity.

Three possibilities of explaining the acidity regulation were discussed: *dilution*, *neutralization* and *diffusion*.

The experimental data indicated that in a Heidenhain pouch stimulated with histamine, the acidity regulation was mainly due to diffusion processes according to the "diffusion theory."

Some inconsistencies of the theory were explained by the fact that the gastric pouch could never be completely emptied but contained a remaining volume, which was thought to interfere with the relation between the acidity and the secretion rate. CHAPTER 10 (page 46).

Total Output of Hydrochloric Acid in Relation to the Secretion Rate.

A simple relation between the secretion rate and the total output of HCl was found both theoretically and experimentally. The relation was considered to be a net result of secretion and diffusion. CHAPTER 11 (page 67).

The Chloride Concentration in the Gastric Juice.

The concentration of chloride in the gastric juice was found to be in accordance with the diffusion theory.

The chloride accumulation in the stomach was discussed and a new theory was proposed as an attempt to explain the phenomenon. The

theory assumed that bicarbonate ions of the plasma were "indiffusible" through the gastric mucosa due to the presence of carbonic anhydrase. In the mucosa the bicarbonate ions were substituted by chloride ions which entered the stomach. — Some experiments with sodium thiocyanate seemed to support the theory. CHAPTER 12 (page 70).

Total Output of Chloride in Relation to the Secretion Rate.

A simple relation between the secretion rate and the total amount of Cl^- was found theoretically and experimentally on the basis of the "diffusion theory." CHAPTER 13 (page 81).

The Excretory Function of the Parietal Cells. Neutral Red Elimination.

It was considered that continuous intravenous injection of neutral red would maintain the concentration of the dye in the blood at a constant level. Under such conditions the relation between the secretion rate and the neutral red concentration in the gastric juice was observed. The lower the secretion rate the higher the concentration of the dye in the gastric juice.

With the same technique the relation between the blood concentration of neutral red and the elimination was also studied. The resulting curves indicated that reabsorption of the dye possibly occurred. The results were discussed. CHAPTER 14 (page 84).

Influence of Enterogastrone on the Parietal Secretion.

An active enterogastrone preparation was found to influence the secretion rate alone and had no *specific* action on the acidity, chloride concentration or neutral red elimination. The depressing effect on the secretion rate was probably not due to any histaminase effect of the enterogastrone. CHAPTER 15 (page 92).

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CHOLINESTERASES

A Study in Comparative Enzymology

By

Klas-Bertil Augustinsson

STOCKHOLM

1948

To my wife

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LIST OF SYMBOLS

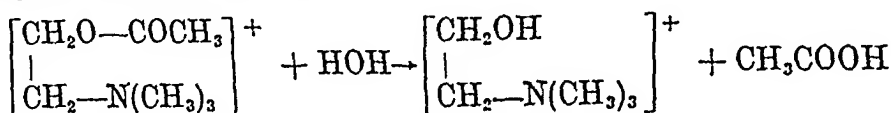
| | |
|----------------------------------|---|
| b_{30} | Enzyme activity (total hydrolysis a_{30} — non-enzymic hydrolysis) expressed as $\mu\text{l. CO}_2/30 \text{ min.}$ |
| k, k_0, k_1 | Velocity constants of destruction of substrate. |
| t | Time in minutes. |
| a | Initial amount of substrate expressed as $\mu\text{l. CO}_2$, that is, the amount of CO_2 evolved at complete hydrolysis. |
| x | Amount of substrate, expressed as $\mu\text{l. CO}_2$, hydrolysed after the time t ; for $t = 30 \text{ min.}$, $x = a_{30}$. |
| $[E]_{\text{tot}}$ | Molar concentration of total enzyme centres. |
| $[E]$ | Molar concentration of free enzyme centres. |
| $[S]$ | Molar concentration of free substrate (= initial concentration); $\text{pS} = -\log [S]$. |
| $[I]$ | Molar concentration of free inhibitor (= total concentration). |
| $[ES], [ES_2]$ | Molar concentration of enzyme-substrate complexes. |
| $[EI], [EIS]$... | Molar concentration of enzyme-inhibitor complexes. |
| K_S, K_{S_1} | Dissociation constants of enzyme-substrate complex ES. |
| K'_S | Apparent dissociation constant of enzyme-substrate complex ES in the presence of a constant concentration of inhibitor. |
| K_{S_2} | Dissociation constant of enzyme-substrate complex ES_2 . |
| K_I | Dissociation constant of enzyme-inhibitor complex EI. |
| v, v' | Observed velocity of enzymic hydrolysis (= b_{30}) without and with inhibitor respectively. |
| V_{max} | Maximum velocity of enzymic hydrolysis occurring at infinite substrate concentration. |
| V_{opt} | Optimum velocity of enzymic hydrolysis occurring at finite substrate concentration when excess of substrate inhibits enzyme activity. |
| pS_{opt} | — \log optimum substrate concentration, at which optimum velocity occurs. |
| pS_1, pS_2 | — \log substrate concentration at $\frac{1}{2} V_{\text{opt}}$. |

"Enzyme chemistry has proved itself repeatedly to be one of the most powerful tools in transforming our approach from pure description of the phenomena of the living cell into scientific analysis."

DAVID NACHMANSOHN
in "Currents in Biochemical Research"
(1946)

GENERAL INTRODUCTION

Cholinesterase — sometimes named acetylcholine esterase — is the enzyme which catalyses the hydrolysis of the acetic-acid ester of choline, the physiologically important acetylcholine. Thus the enzyme catalyses the reaction:



The decrease of free energy in this process is great and the reaction is essentially irreversible. The resynthesis of acetylcholine in the organism is most probably not catalysed by cholinesterase; in this reaction another enzyme, choline acetylase, takes part.

An enzyme, which hydrolyses only choline esters, is a *specific cholinesterase* and such an enzyme has been proved to exist in the animal organism. The physiological substrate of this specific cholinesterase is acetylcholine which is the only choline ester chemically identifiable in animal tissues. Non-specific cholinesterases can hydrolyse other esters in addition to choline esters, but the latter are split at the highest rate. Proofs have been offered that at least one such *non-specific cholinesterase* occurs in the animal organism. The physiological function of this type of cholinesterase, however, is more obscure, and the substrate not known. Generally, both types of cholinesterase split acetylcholine at higher rates than other choline esters. Also other properties, not found with ordinary esterases, are common to the two types of cholinesterases. For instance, both these enzymes are inhibited by very small amounts of physostigmine, so that it is justifiable to separate them from other esterases. Unspecified esterases may split all sorts of esters at the same rate or even at higher rates than acetylcholine and other choline esters.

The cholinesterases belong to a group of esterases called *azol-esterases* (GLICK, 1942) which hydrolyse nitrogen-alcohol esters. Other examples of enzymes belonging to this group are morphine

esterase, the tropine esterases, and procaine esterase. As cholinesterase, they are present in serum, and, most probably, they are not identical with the serum cholinesterase.

General reviews of cholinesterase have been reported by, amongst others, AMMON (1935, 1940), HANSKE (1941), GLICK (1941 a), and WERLE (1943). A voluminous literature has appeared on the various aspects of cholinesterase, most of these articles dealing with the physiological significance of the enzyme. Much literature has appeared on cholinesterase in vertebrate as well as invertebrate animals, in normal as well as in pathological states. The enzyme has also been studied chemically and this aspect has evoked particular interest in late years. The present investigation is intended to add to our knowledge of the chemistry and especially the specificity of cholinesterase activity.

It will be noted that the interest in cholinesterase is based upon its physiological significance and the chemical properties of the enzyme are intimately connected with its physiological functions. It appears desirable therefore to open the following discussion on the chemistry of cholinesterase with a short review of its physiological significance (Chapter I), including data about its distribution in various animal tissues. This is followed by a summary of the previous literature on the chemistry of cholinesterase (Chapter II). Part II deals with the methods (Chapter III) and materials (Chapter IV) used in the investigations, the results of which are described in Part III. After the discussion of the non-enzymic hydrolysis of the various substrates used (Chapter V) a detailed account is given of the cholinesterase activities of various tissues compared with other esterase activities (Chapter VI). Chapter VII involves a comparative study of the kinetics of the enzymic hydrolysis of choline esters and is followed by a discussion on the relationship between reaction rate and enzyme concentration (Chapter VIII). A detailed study of the activity-substrate concentration relationships of various cholinesterase-substrate systems is presented in Chapter IX, as is also the influence of various substances on those relationships. Chapter X discusses the effect of choline on the cholinesterase activity under various circumstances. The influences of some further compounds on the enzyme activity are reported in Chapter XI whilst the hydrolysis of mixtures of substrates are studied in Chapter XII. In conclusion, the paper ends with a Discussion (Chapter XIII) and a General Summary.

PART I

CHAPTER I

THE PHYSIOLOGICAL SIGNIFICANCE OF CHOLINESTERASE

Cholinesterase (ChE) and its substrate acetylcholine (ACh) hold an important position in the physiology of the nervous system. They are essential to the metabolism of all nerve cells and in addition, the ChE system is said to be an integral part of the mechanism responsible for the development and propagation of the action potential.

A. THE ACETYLCHOLINE-CHOLINESTERASE SYSTEM AND THE TRANSMISSION OF NERVE IMPULSES

The interest in acetylcholine lies in the characteristic effect of that substance on the organism. ACh produces a response in effector cells similar to that obtained by the stimulation of so-called cholinergic nerves (p. 4).

For many years following the synthesis of ACh in 1867 by BAEYER, the interest in this substance was purely a chemical one. The pharmacological properties of ACh were examined in more detail by DALE (1914) who also observed the evanescence of the effect which is characteristic of all actions of ACh: "... it seems not improbable that an esterase contributes to the removal of the active ester from circulation, and the restoration of the original condition of sensitiveness".

The responses to ACh in the organism are very transient, due mainly to the enzymic hydrolysis of the substance to acetic acid and choline whose pharmacological effect is 100 000 times less than its acetyl derivate. This hydrolysis is caused by the enzyme *cholinesterase*. The enzymic action is selectively inhibited by small doses of physostigmine. A dorsal muscle of the leach treated with physostigmine contracts powerfully in the presence of minute traces of ACh ($1 : 10^6$). This is the usual test for detection of ACh (MINZ, 1932).

The action of ACh on the heart is particularly characteristic producing the same effects as stimulation of the vagal fibres. It was research along these lines that established the first real proof of the chemical mediation of nerve impulses and laid the foundation for the hypothesis of "neurohumoralism". This hypothesis postulates that nerves act by liberating from their terminals chemical substances and in this way transmit the nerve impulse to their effector organs. An interesting review of this hypothesis is to be found in a lecture by OTTO LOEWI (1945), the discoverer of the "Vagusstoff".

LOEWI was the first to establish the "humoral" transmission of nervous action. This he did in 1921 in a decisive manner by simple but quite convincing experi-

ments. When stimulating the vagus nerve of a frog's heart perfused with RINGER's solution and allowing the perfusion fluid to pass into a second heart, a substance was noted to be liberated from the first heart which slowed the rate of the second one as if the vagus to this one were stimulated. This mediating substance was called "Vagusstoff" and subsequently LOEWI and NAVRATIL (1926) assumed it to be identical with acetylcholine. LOEWI also detected an "Acceleransstoff" similar to adrenaline which was liberated in the perfusion fluid when the accelerator nerve was stimulated.

Subsequent elaboration of LOEWI's hypothesis has established the identity of the parasympathetic mediator as acetylcholine. Moreover, ACh has been shown to play a far more important rôle than the mediator of impulses for post-ganglionic parasympathetic nerves. Thus all autonomic preganglionic nerve impulses, whether parasympathetic or sympathetic, are accomplished by the release of ACh at the ganglionic synapses. ACh is also the mediator of nerve impulses which cause the secretion of adrenaline from the cells of the adrenal medulla, sweating, and vasodilatation.

The fact that the anatomical classification of the autonomic nervous system into sympathetic and parasympathetic divisions does not coincide with their physiological functions caused DALE to propose a new subdivision of the autonomic system into *adrenergic* and *cholinergic* systems. Adrenergic nerves include all fibres whose impulses act through the release of an adrenaline-like substance (sympathin) and consist of postganglionic sympathetic nerves. All other autonomic nerves are cholinergic, the impulses of which act through the release of ACh.

Since evidence was presented that ACh might mediate the nerve impulses from motor nerves to striated muscles or from neurone to neurone in ganglia, many neurophysiologists have been inclined to believe that the same mechanism predominates in the central nervous system for synaptic transmission. Particularly interesting in regard to this complex problem are the investigations of NACHMANSOHN and his co-workers (1939-1947) on the ChE activity in the central nervous system (p. 9). These and other researches have pointed to the possibility of chemical mediation in central synaptic transmission.

ChE hydrolyses ACh and this is most probably the rôle that it plays in the organism. Thus ChE is said to destroy ACh immediately after transmission has occurred and within the refractory period of the muscle or ganglion cells. High concentrations of ChE are present at motor end-plates, at all ganglionic synapses, and at all synapses of the central nervous system (cf. ARMSTRONG, 1945). The distribution of the enzyme is discussed more fully below (p. 6).

The antagonists of the hypothesis of chemical transmission of nerve impulses are the electrobiologists who postulate an exclusively electrical mechanism for the transmission; chemicals liberated are thought to act as regulators only. ECCLES, LORENTE DE NÓ and others have doubted that sufficient quantities of ChE were produced in the required time, i.e., the refractory period, to destroy the ACh liberated (see p. 8). It is difficult to believe, however, that the liberation of ACh by nerve impulses which has been proved beyond dispute and the relatively high concentration of ChE at the precise place where it can play an essential rôle in such a chemical mechanism, are purely incidental and without physiological significance.

In 1937, VON MURALT suggested that ACh is liberated in the axon as well as at the nerve ending and he was able to demonstrate that this liberation occurs only during excitation. ACh is considered as an "Aktionssubstanz" or a compound connected with nervous activity. This proposal of VON MURALT has been elaborated and extended by NACHMANSOHN (review: NACHMANSOHN, 1943, 1945, 1946, 1947; NACHMANSOHN & ROTHENBERG, 1946; FULTON & NACHMANSOHN, 1943; FULTON, 1945). He proposed this new concept of the rôle of ACh as a compromise between the opposing chemical and electrical schools of thought. It is based mainly on the results obtained from investigating the enzyme ChE, that is, the extremely active enzyme system required for a rapid inactivation of free ACh (cf. NACHMANSOHN,

1940 d; BULLOCK, NACHMANSOHN & ROTHENBERG, 1946; BULLOCK, GRUNDFEST, NACHMANSOHN & ROTHENBERG, 1947; GRUNDFEST, NACHMANSOHN & ROTHENBERG, 1947). Doubt, criticism, and frank disagreement have been levelled at NACHMANSOHN's hypothesis, but this paper is not the place to discuss them.

GESELL and HANSEN (1945) based a humoro-electrotonic hypothesis on the electrogenic action of ACh at the site of its liberation, and they considered the inhibiting effect of physiological increase in cH on ChE an important mechanism for regulating the activity of nerves. Carbon dioxide is said to stimulate the neuron by decreasing the rate of destruction of ACh by ChE, and thus indirectly increasing the electrotonic current.

It is not yet certain whether substances other than ACh take part in cholinergic transmission. KUH, WIELAND and HUEBSCHMANN (1939) have assumed that aneurine in the form of its acetyl ester might be a mediator of nerve impulses. Acetylaurine is enzymically split by the ACh-hydrolysing enzymes of brain (MASSART & DUFAY, 1939 b) and of blood serum (AUGUSTINSSON, 1944); this hydrolysis will be discussed more fully in Part III of this paper. Previously, VON MURALT and his co-workers (review: VON MURALT, 1945) designated an active form of aneurine (not acetylaurine) as the second action substance ("zweiter Vagusstoff") in cholinergic nerves besides ACh. Additional action-substances have also been isolated by these authors whilst other authors have assumed that histamine plays a rôle as a chemical mediator. Moreover, thyramine has been suggested a chemical excitator in some low animal species (see MOORE, 1947).

B. OTHER CHOLINE DERIVATIVES AND THEIR PHARMACOLOGICAL PROPERTIES

Several choline derivatives (almost 300) have been synthesised in an attempt to find the basis for the susceptibility of ChE to hydrolytic destruction. The esters of choline are usually enzymically hydrolysed by blood and tissues. The ethers of choline are not affected by ChE. Betaine is practically inert as regards its pharmacological actions. More details about the enzymic destruction of choline derivatives are given in Chapter II (p. 16). Acetyl- β -methylcholine and carbaminoylcholine are alone satisfactory for clinical use.

The pharmacological properties of acetyl- β -methylcholine (called mecholyl in the form of its chloride) are qualitatively similar to those of ACh. The former drug is less readily split by ChE (Part III), which explains the longer duration of its action. Carbaminoylcholine (used in the form of its chloride under the names of doryl and lentin) is much more stable in body fluids than ACh and mecholyl; it is not destroyed by ChE (see Part III) and its action is neither intensified nor prolonged by physostigmine or prostigmine.

C. CHOLINESTERASE

1. Historical Note

DALE, as early as 1914, suggested that an enzyme was present in the blood which brought about the destruction of ACh. In 1925, ABDERHALDEN and PAFFRATH pointed to the existence of such an enzyme in the small intestine of horse and pig. LOEWI and NAVRATIL demonstrated in 1926 the existence of an enzyme in extracts prepared from heart muscle which inactivated the "Vagusstoff". They interpreted this inactivation as the hydrolysis of an unstable choline ester which was inhibited by physostigmine; this ester was ACh. About the same time, PLATTNER (1926) noticed the great ACh-hydrolysing effect of blood and PLATTNER *et al.* (1928) explained this inactivating effect of the blood as a non-specific surface

catalysis by the erythrocytes. CLARK (1927) confirmed the conclusion of LOEWI and NAVRATIL that the destruction of ACh by heart tissue is due to an enzyme. ENGELHART and LOEWI (1930) explained the action of blood on ACh by suggesting the existence of an esterase which was completely inhibited by minute amounts of physostigmine, whilst MATTHES (1930) showed that the conditions for the inactivation of ACh were consistent with the action of an esterase. PLATTNER and HINTNER (1930) were the first to show that the enzyme is present in nearly all animal tissues. In 1932 STEDMAN, STEDMAN and EASSON prepared from horse serum an enzyme which was considered to be a specific esterase for ACh; this they called *choline-esterase*¹, as it was shown to be capable of splitting other choline esters. STEDMAN, STEDMAN and WHITE (1933) compared the ChE activities of the blood sera from various species. Later investigations, however, do not support the view that the enzyme prepared by STEDMAN *et al.* was really a specific ChE, a fact which will be discussed more fully below (p. 18).

2. Distribution of Cholinesterase

The distribution of ChE has been studied in considerable detail in connection with the physiological significance of the ACh-ChE-system. ChE has been found in almost all multicellular animals, it was present in all vertebrates and in most invertebrates investigated. In the plant kingdom, on the other hand, the enzyme has not yet been found (as regards fungi, see OURY & BACQ, 1938).

A summary of the distribution of ChE in different tissues is given below, vertebrate tissues being discussed first.

a) *Blood*. Previous investigations about the distribution of ChE in the blood of vertebrates have given the following results. The enzyme concentration is the same in the venous and arterial blood (MAHAL, 1938). Serum and oxalate plasma have the same activity. The leucocytes do not split ACh, which is hydrolysed at the same rate by reduced and oxidised erythrocytes (FEGLER, KOWARZYK & SZPUNAR, 1937). STEDMAN, STEDMAN and WHITE (1933) could not detect any activity at all in serum obtained from ox, frog, rabbit, or rat, but as was shown at first by PLATTNER *et al.* (1928) whole blood from these species did have ChE activity. In man, ox, sheep, goat, and rabbit, the blood cells contain more ChE than serum or plasma. In horse, dog, cat, duck, and fowl, the distribution is reversed. The high ChE activity of horse serum in contrast to that of ox serum has been used by VINCENT and BROCA (1946) to differentiate the two sera from each other. Whole and haemolysed blood are said to contain the same amount of ChE (AMMON & VOSS, 1935; cf. DOMINI & COLOMBINI, 1938; LITTLE, 1939). The enzyme activities of the blood of various vertebrates have also been studied by, amongst others: AMMON (1933), STEDMAN and STEDMAN (1935 a), HALPERN and CORTEGGIANI (1935), INGVARSSON (1935), KAHANE and LÉVY (1936 a), KWIATKOWSKI (1936), VEREBÉLY (1936), EASSON and STEDMAN (1937), HALL and LUCAS (1937 b), GLICK (1938 a), PERUZZI

¹ STEDMAN *et al.* also spelled the new word in this way, but later authors have occasionally employed it as a single word: *cholinesterase*. Almost exactly half of the papers in English use *cholinesterase* whilst the others use *choline esterase* or *choline-esterase*. Latterly the single word has gained in popularity and will therefore be employed in this paper.

(1938), BENDER (1939), GLICK, LEWIN and ANTOPOL (1939), and FRIEND and KRAYER (1941). The present investigation also deals with the ChE activity of the blood of various vertebrates (Part III).

CROFT and RICHTER (1943) and BRAUER and ROOT (1945) have supposed that blood-cell ChE is adsorbed on the surface of the erythrocytes. This has been confirmed by the present author and will be discussed more fully in Part III (p. 57). CLINE, JOHNSON and JOHNSON (1947) found, on the other hand, that the ChE activity did not parallel the cell count or total blood haemoglobin.

In 1940 the identity between "serum-ChE" and "erythrocyte-ChE" was disputed by ALLES and HAWES (also HAWES & ALLES, 1941) since they found that the ACh-hydrolysing enzymes in blood serum and red blood cells were not identical. These findings were later confirmed by RICHTER and CROFT (1942), MENDEL and RUDNEY (1943 a), ZELLER and BISSEGER (1943), AUGUSTINSSON (1944, 1945), GLASSON (1944, 1945), NACHMANSON and ROTHENBERG (1945), and SCHAEFER (1947). A non-specific (*s*-type, pseudo-) ChE seems to be present in sera, but is absent in the blood of certain animals. A specific (*e*-type, true) ChE is present in the erythrocytes (in certain cases in the plasma) (MENDEL, MUNDELL & RUDNEY, 1943; MENDEL & RUDNEY, 1944 b). These observations will be discussed more fully below (p. 18 and Part III).

ChE is also present in the lymph, though in considerably smaller amounts (TSUJI, 1932; KWIATKOWSKI, 1936; GLICK, LEWIN & ANTOPOL, 1939; FORBES, OUTHOUSE & LEACH, 1940; FRIEND & KRAYER, 1941). Ascitic fluid and pus have also been shown to split ACh (GINSBERG, KOHN & NECHELES, 1937).

The ChE concentration in the blood of man has been studied in considerable detail in order to correlate if possible the enzyme activity with various diseases (see p. 14). However, the results are not easy to interpret because of the marked variation in the ChE activity of normal human beings. The esterase values of one and the same individual also appear to vary considerably when estimated over a long period (STEDMAN, STEDMAN & WHITE, 1933; WHITE, 1933; AMMON & VOSS, 1935; VAHLQUIST, 1935; VEREBÉLY, 1936, 1937; MILHORAT, 1938; MAHAL, 1938; BERGER, 1939; JELLINEK & LOONEY, 1939; FABER, 1941; FERRONT & BALLI, 1944; cf. ABDON & UVNÄS, 1937; HALL & ETTINGER, 1937). According to HEDR (1944) the serum-ChE concentration decreases with age. In contradistinction to other animals the serum-ChE concentration of guinea pigs shows seasonal variations (WATTENWYL, BISSEGER, MARITZ & ZELLER, 1943; WATTENWYL & ZELLER, 1944; HERSCHBERG, FROMMEL & PRIQUET, 1944). In addition, a geographic or climatic variation has been suggested in the ChE activity of human serum (LACKEY & SLAUGHTER, 1939, 1942).

Normal physiological processes have no influence on the ChE in serum (HALL & LUCAS, 1937 a; SOBOTKA & ANTOPOL, 1937; MAHAL, 1938; DOMINI & COLOMBINI, 1938; PERUZZI, 1938; BERGER, 1939; HUIDOBRO & CROXATTO, 1939; ZELLER & BIRKHÄUSER, 1940; DROUET, VERAÏN & FRANQUIN, 1942; FABER, 1941, 1943 a, b). VILLASANTE (1941) has stated that the temperature of the body influences ChE, an observation which was not confirmed by LACKEY and SLAUGHTER (1942). Increased serum ChE activity during muscle exercise, found by CROFT and RICHTER (1943) (also CROXATTO *et al.*, 1939; HUIDOBRO, GUZMÁN & ANDIA, 1943) has not been confirmed by STONER and WILSON (1943). The changes in the serum-ChE activity during nervous stimulation have been investigated by H. and R. CROXATTO *et al.* (1939, 1940, 1941).

It has previously been held that no difference exists between the serum-ChE concentration in men and women. Observations, made by ZELLER and his co-workers, argue against this (BIRKHÄUSER & ZELLER, 1940; ZELLER, BIRKHÄUSER,

WATTENWYL & WERNER, 1941; WATTENWYL, BISSEGGER, MARITZ & ZELLER, 1943). After puberty the ChE level is higher in men than in women, but particularly low values were found in the serum of pregnant women (also found by BUTT, COMFORT, DRY & OSTERBERG, 1942) though this was not confirmed by HERSCHBERG, GEISENDORF and PIQUET (1947). It is suggested that the ChE level of human serum (and of liver) is affected by the female as well as the male hormones, but other results have been obtained with the lower animals. Thus BEVERIDGE and LUCAS (1941) found more ChE in the serum of mature female rats and mice than in that of mature males. According to MUNDELL (1944) this difference is ascribed to the "pseudo-ChE". CARIDROIT, KASWIN and SERFATY (1945) found that the ChE activity of the plasma of cocks was about 50 per cent greater than that for hens of the same breeds. More details about the sex difference in the ChE levels of rats have been published recently by SAWYER and EVERETT (1946, 1947) and EVERETT and SAWYER (1946 a, b).

Regarding the action of parturition on the serum ChE, NAVRATIL (1939 a) in opposition to VAHLQUIST (1935) and HERSCHBERG *et al.* (1944 a) has shown that after the birth of the child the concentration of the serum ChE decreases in most cases. The enzyme concentration is said to be higher in the blood of the mother than in that of the umbilical cord (AMMON, 1935; NAVRATIL, 1937 b).

b) *Nervous system.* It has now been established that the ChE in all nerve tissues is either exclusively or predominantly specific for ACh (MENDEL & RUDNEY, 1943 b; LANGEMANN, 1944 a; NACHMANSOHN & ROTHENBERG, 1945; SAWYER & EVERETT, 1947). More details about the type of ChE present in brain will be found in Part III.

A major difficulty in the chemical mediation theory is how ACh, released by the nerve impulse, can be rapidly removed within the brief refractory period of skeletal muscles and ganglia. An answer to this criticism is given by a series of well planned experiments on the enzyme ChE, carried out in the first place by NACHMANSOHN and his co-workers. If ACh is the mediator of nerve impulses it would have to be destroyed during the refractory period. The ChE concentration, however, is surprisingly low in striated muscles (cf. Chapter VI). The time of hydrolysis by a muscle is 60 000 to 40 000 times longer than the refractory period. In the case of the frog sartorius, the ChE concentration is 300 per cent higher in the part containing nerve endings than in the nerveless pelvic end, also found for mammalian and other animal muscles. This difference is attributed to the high concentration of the enzyme at the nerve endings (MARNAY & NACHMANSOHN, 1937 a, b, c, g, 1938; MARNAY, MINZ & NACHMANSOHN, 1937; COUTEAUX & NACHMANSOHN, 1940; FREDERICQ, 1937; FENG & TING, 1938; HELLAUER, 1939; HELLAUER & UMRATH, 1939; PIGHINI, 1939 d). This concentration is high enough to support the assumption that ACh is the transmitter of impulses across the neuromuscular junction. The evidence presented for the high concentration of ChE at motor end-plates has been critically reviewed by CLARK, RAVENTÓS, STEDMAN and STEDMAN (1938), LITTLE and BENNETT (1940), and GLICK (1941 a).

As regards the sympathetic ganglia, GLICK (1937 b, 1938 d), working with the superior cervical ganglion, suggested that the concentration of ChE may be more concentrated at the synapses. The ChE activity of this ganglion has also been studied by BRÜCKE (1937), CROXATTO *et al.* (1940), and COUTEAUX (1942). Evidence in support of GLICK's theory was presented by NACHMANSOHN (1938 b, f, 1939 a). It was calculated that the concentration of ChE at the synapses was more than sufficient to destroy within the refractory period, the ACh liberated by a maximal preganglionic stimulus and was capable of causing a postganglionic discharge. MENDEL and RUDNEY (1944 b), also SAWYER and HOLLINSHEAD (1945), have stated that a mixture of specific and non-specific ChE is present in the superior cervical ganglion of the cat.

In the central nervous system, ChE is much more abundant in the gray matter which contains the cell bodies and synapses; the ChE values, however, vary considerably in different species and in the different parts of the brain (NACH-

MANSOHN, 1937, 1938 b, c, d, h, 1939 a; PIGHINI, 1938, 1939 a; LANGEMANN, 1942; RICHERT & SOHNARRENBURGER, 1942; EGAÑA, 1946) and of the spinal cord (MARTINI & TORDA, 1938 c; NACHMANSOHN & HOFF, 1944). Consistently constant values are found for the same part of the same species. Likewise there is a close resemblance between the distribution of ACh and ChE (WELSH & HYDE, 1944). All these results support the view that the same enzyme mechanism exists at central synapses as that found at motor end-plates and at ganglionic synapses.

The ChE values in brain are the highest for adult human beings, the lowest values being obtained in children (BIRKHÄUSER, 1940). The values differ in men and animals (PIGHINI, 1938; ZELLER, BIRKHÄUSER, MISLIN & WENK, 1939). The ChE activity of the brain of various species has also been studied by STEDMAN and STEDMAN (1935 a), KUHN and SURLES (1938), PERUZZI (1938), GLICK, LEWIN and ANTOPOL (1939), and by the present author (Chapter VI).

The high concentration of ChE in nerve fibres and the still higher one in the synaptic region, is particularly noticeable in fibres without myelin sheaths, e.g., the fibres of the sympathetic chain. In the myelin sheath itself the concentration seems to be negligible. The difference in enzyme concentration between nerve fibres and synapses is due in part to the increased surface occurring inside the ganglion by the extensive end-arborisation of the pre-ganglionic fibres. ChE is supposed to be concentrated exclusively at the neuronal surface of the axon; no enzyme is found in the axoplasm. Evidence for such a localisation of ChE has been obtained in experiments on the giant axon of *Loligo* (squid) (BOELL & NACHMANSOHN, 1940; cf. NACHMANSOHN & MEYERHOF, 1941; SAWYER, 1946).

BULLOCK, GRUNDFEST, NACHMANSOHN and ROTHENBERG (1946, with STERLING; 1947) have observed a striking parallelism between enzyme activity and action potential, which means that a correlation exists between conduction and ChE activity (cf. p. 12). The changes of ChE activity in nervous tissues under the influence of direct current have been investigated by MINAJEV (1942) and by BABSKY and MINAJEV (1944).

ChE is found also in the cerebrospinal fluid (PLATTNER & HINTNER, 1930; ALTENBURGER, 1937; PINOTTI & TANFANI, 1939; BENDER, 1939; REISS & HEMPHILL, 1948; cf., however, STEDMAN & STEDMAN, 1935 a; VAHLQUIST, 1935). The activity is equivalent to that of about 1 per cent blood serum. The cerebrospinal fluid seems to contain a specific ChE and an "aliesterase" (GLASSON & MUTTRIX, 1946).

c) *Muscles*. The ChE activity of striated muscles has been discussed to some extent in relation to the high enzyme concentration at the motor end-plates (p. 8). According to LANGEMANN (1944 a) the enzyme in striated muscles is of pure *c*-type (specific ChE) which has also been shown by NACHMANSOHN and ROTHENBERG (1945).

The ChE concentration is higher in animals that move rapidly than in those that are less active (MARNAY & NACHMANSOHN, 1937 d; MARNAY, 1938 a; LINDEMAN, 1945) (cf. p. 66). The concentration is proportional to the chronaxie (MARNAY, 1938 a).

A muscle loses its ACh dépôt on denervation, the susceptibility to ACh rising. This has been attributed to a decrease in the ChE concentration of the denervated muscle. MENG (1940), however, did not find any connection between the enzyme activity in muscle and the susceptibility to ACh. MARTINI and TORDA (1937, 1938 d) (TORDA & MARTINI, 1938; cf. FENG & TING, 1938) have shown that the ChE concentration in denervated *m. gastrocnemius* decreases with the time after denervation (cf. LEIBSON, 1939 b; also STOERK & MORPETH, 1944), found also in denervated muscle of *Amblystoma* (SAWYER, 1943 b). MARNAY and NACHMANSOHN (1937 f, g), on the other hand, have found an increase in the ChE concentration after denervation (guinea pig); they supposed that it was not the nerve fibres, but the nerve cells which were sensitive to the ACh liberated on nervous stimulation. These cells are, in fact, very much increased in denervated muscles. COUTEAUX and NACHMANSOHN (1938, 1940) explained the increase of ChE to be only apparent as a result of the simultaneous muscular atrophy; in reality the absolute ChE concentration decreases (cf. COUTEAUX, 1942; RYBINOWSKAJA, 1940 b; PIROLI, 1942 c).

TABLE 1. *Various Vertebrate Tissues Investigated for their Cholinesterase Activity*

Estimations for each organ have been carried out on a variety of animals. Where only one or two have been determined this is stated in parentheses. The data about the type of ChE have been taken chiefly from the paper by SAWYER and EVERETT (1947), also from the papers by LANGEMANN, NACHMANSOHN *et al.*, MENDEL *et al.*, etc., published after 1944. I: specific ChE; II: non-specific ChE.

| Tissues (Animals) | ChE | | References |
|---|-----------|---------|--|
| | Ac-tivity | Type | |
| Heart | ++
+ | I
II | 347, 162, 496, 116, 477, 217, 232,
26, 576, 206 |
| Lung | ++ | | 496, 232, 329, 328 |
| Salivary glands | + | I | 352, 477, 232 |
| Saliva | +++ | II | |
| Stomach (pig, frog) | — | | 496, 77 |
| Gastric juice (man, rabbit) | ++ | | 217, 218, 232 |
| Pancreas | ++ | I | 77 |
| Pancreatic juice | ++ | II | 496, 14, 212, 354, 358, 477, 232,
326 |
| Liver | ++ | II | 212 |
| Gall bladder, bile | + | I | 496, 14, 245, 366, 147, 477, 232,
211, 307 |
| Intestine | +++ | II | 496, 321, 232 |
| Spleen | — | | 1, 347, 496, 245 232, 326, 110, 307 |
| Kidney | ++ | I | 496, 358, 232 |
| Adrenal glands | + | II | |
| Bladder, ureters (calf).... | + | I | 496, 366, 477, 232, 211, 327, 328 |
| Urine (man) | + | II | |
| Testis (man, pig) | ++ | I | 496, 477, 232, 28 |
| Sperm, prostate, seminal vesicles (man) | + | II | |
| Spermatc fluid from shark (<i>Scyllium</i>) | ++ | | 180 |
| Ovary, oviduct (man, pig) | — | | 496, 15, 618, 212 |
| Uterus | + | | 232, 326 |
| Placenta (man) | (+) | | 686 |
| Milk (cow) | +++ | | 308 |
| Thyroid gland | + | I | 232, 326, 327 |
| Carotid body (cat) | ++ | II | |
| Thymus | + | I | 496, 366, 232 |
| Eye: various parts of | ++ | II | |
| Ear: perilymph (pigeon) .. | + | I | 466, 609, 666 |
| Skin (man, cat) | — | | 496 |
| Hibernating gland (cat) .. | + | I | 496, 232, 326, 327 |
| Red bone-marrow (cat)... | ++ | II | |
| | + | I | 271; cf. 12 |
| | ++ | II | |
| | + | I | 496, 232, 326, 327, 328 |
| | ++ | I | 496, 617, 94, 232, 442, 32, 263,
24, 653 |
| | + | II | |
| | ++ | I | 368 |
| | + | I | 604, 687 |
| | + | I | 554 |
| | ++ | II | |
| | + | I | 554 |
| | + | II | |

Another explanation for these findings is postulated by SAWYER (1946). Wallerian degeneration of nerves *in vivo* is accompanied by a 60 per cent loss of their specific ChE content. Further loss of enzyme fails to occur. The non-specific ChE is unaffected by the operation while total ACh-hydrolysing capacity, dependent on two enzymes, drops 30 per cent. *In vitro* experiments, however, indicate a decline of both specific and non-specific ChE, as was shown by BORLL (1945 a).

The ChE activity of muscles in various animals does not necessarily change with age (NACHMANSOHN, 1939 c; RZABINOWSKAJA, 1940 a). Such differences may be due to the fact that the relative mass of the muscle and the nerve endings are not the same.

SEIDLITZ (1938) and RUNCAN (1940) found increased activity in muscle after contraction by tetanic stimulation; other authors (LISSÁK, NAGY & PÁSZTOR, 1942; VINCENT & JULIEN, 1941) found a decrease in activity. After the injection of tetanus toxin the concentration of ChE is increased in m. gastrocnemius, decreased in m. tibialis anterior (MARTINI, TORDA & ZIRONI, 1939). According to the same authors (MARTINI & TORDA, 1938 b), the enzyme activity decreases in muscles after destruction of the cerebellum. Section of the lumbosacral cord leads to a small decrease of the enzyme concentration of m. gastrocnemius (MARTINI & TORDA, 1938 a, c; TORDA & MARTINI, 1938). The centres in the spinal cord are said to be of great importance in the maintenance of the ChE concentration of muscle. The muscle ChE also decreases after adrenalectomy (STORR & MORPETH, 1944).

d) *Other vertebrate tissues.* The ChE activities of other vertebrate tissues, not discussed above, are presented in Table I which shows, where it has been determined, the type of enzyme present. The earlier data concerning the ChE activity of various tissues furnish no information as to whether specific or non-specific ChE, or both, are responsible for the ACh-hydrolysis in the tissue. The first account dealing with the amount of specific and non-specific ChE contained in various tissues of a single species is published by AUGUSTINSSON (1946 c) in the case of *Helix pomatia* and by SAWYER and EVERETT (1947) in the case of the rat.

The following findings are of especial note. In the *hearts* of mammals (and those of frogs) the ChE concentration is higher in the auricles than in the ventricles (ENGELHART, 1930; ANTOPOL *et al.*, 1939 a). *Salivary glands*, especially the parotid gland, contain much ChE. GLICK (1938 a, b) has applied a micromethod for assay of ChE to the study of the distribution of the enzyme in the *gastric mucosa*. The *pancreas* is said to play an important rôle in the system that regulates the ChE activity of blood serum; some secretion (not insulin) decreases the hydrolytic action (SANTENOISE & BOVER, 1941). Several investigations indicate that serum ChE is probably synthesised in the *liver* (p. 14). In the *adrenal gland* the activity is relatively higher in the medulla than in the cortex (ANTOPOL & GLICK, 1940; LANGEMANN, 1942). The *thymus* has a very high ChE activity. A detailed study of the distribution of ChE in various parts of the *eye* has been performed by BRÜCKNER (1943). From the study of the effect of intravenous injection of purified ChE on the pupillary light reflex, MENDEL and HAWKINS (1943) concluded that ACh plays an essential rôle in nerve impulse transmission to the sphincter pupillae (cf. SACHS & HEATH, 1940).

The present author has studied the types of ChE present in various vertebrate tissues. The results of these investigations will be discussed in Part III.

e) *Snake venom.* The presence of ChE in snake venom was first observed by IYENGAR, SEHRA, MUKERJI and CHOPRA (1938) and later confirmed by GHOSH and his co-workers (GHOSH, DUTT & CHOWDHURY, 1939; GHOSH, 1940; GHOSH & CHOWDHURY, 1941) and by CHOPRA and CHOWHAN (1940). The venoms of the species of the Colubridae possess marked ChE activity, while the venoms of the species of the Viperidae show no such activity (ZELLER, 1947; cf. KASWIN & SERFATY, 1945).

The ChE of cobra venom is inhibited by physostigmine (IYENGAR *et al.*, 1938; BOVET & BOVET, 1943). A purified enzyme preparation has been obtained by CHOWDHURY (1942). IYENGAR *et al.* (1938) supposed ChE to be identical with neurotoxin, a fact not confirmed by GHOSH and his associates. According to ZELLER and MARITZ (1945) and ZELLER (1947) the ChE of snake venom behaves like the e-ChE (specific ChE) of mammalian tissues. In addition to ACh, other esters containing acetyl groups and having low molecular weights, are hydrolysed by cobra venom and therefore NITTI (1947) proposes calling such an enzyme "acetylase".

The *bee venom* has not been investigated regarding its ChE action, but will be discussed later on in this paper. (Chapter VI).

f) *Electric organs*. There are three known species with powerful electric organs (*Electrophorus (Gymnotus) electricus*, *Malapterurus electricus*, *Torpedo marmorata*) and several others with weak electric organs. These organs have the highest concentration of ChE found in any tissue (MARNAY, 1937). NACHMANSOHN and LEDERER (1939 a, b) and more recently ROTHENBERG and NACHMANSOHN (1947) prepared highly purified ChE preparations from the electric organs of *Torpedo* and *Electrophorus* respectively, and studied the biochemistry of the enzyme. NACHMANSOHN (1940 d) based a discussion of the physiological significance of ChE on the studies of the enzyme in the electric organs. Detailed studies of the ChE concentration in relation to the electromotive force were carried out with *Electrophorus* by NACHMANSOHN and his co-workers (NACHMANSOHN, 1940 c; NACHMANSOHN, COATES & COX, 1941; NACHMANSOHN, COX, COATES & MACHADO, 1942; NACHMANSOHN, COATES & ROTHENBERG, 1946; cf. NACHMANSOHN & MEYERHOF, 1941). There seems to be a close parallelism between voltage per cm., number of electric plates per cm., and concentration of ChE. According to NACHMANSOHN and ROTHENBERG (1945), the ChE activity in the electric organs is ascribed to a specific ChE.

g) *Invertebrates*. A review of the physiology of the nervous system of invertebrates is reported by PROSSER (1946); it contains a summary of the data on ChE content of invertebrate tissues, also given by AUGUSTINSSON (1946 b).

In the Protozoa neither ChE nor ACh are to be found. As a rule, Coelenterata do not show any ChE activity; the enzyme is found in the hydrozoans and anthozoans, but is lacking in the sponges, scyphozoans, and ctenophorans. *Tubularia*, a hydrozoan coelenterate and one of the lowest animals to possess a nervous system, contains specific ChE (BULLOCK, GRUNDFEST, NACHMANSOHN & ROTHENBERG, 1947; cf. BULLOCK & NACHMANSOHN, 1942). In worms, considerable quantities of the ACh-hydrolysing enzyme are often present. Species from most classes of flatworms, roundworms, and segmental worms have been studied. A particularly high esterase content was found in the dorsal longitudinal muscle of the flatworm, *Cerebratulus lacteus* (SMITH, JACKSON & PROSSER, 1940). The blood of worms, on the other hand, seems to have no activity (cf. p. 62). Among the Crustacea, the lobster and crayfish in particular have been investigated, but other species have also been examined. The enzyme is to be found in muscles and the nervous system, but is lacking in the blood. This is also the case with the King crab (*Limulus*) (SMITH & GLICK, 1939), spiders, and insects. The ChE activity of insect central nervous system has been studied especially by MIHALONIS and BROWN (1941), MEANS (1942), RICHARDS JR. and CUTKOMP (1945), and TOBIAS, KOLLROS and SAVIT (1946); acetyl- β -methylcholine is said to be hydrolysed at a higher rate than ACh (cf. Chapter VI). Considerable amounts of ChE are found in the blood of the molluscs, this activity is lowest in the mussels, a little higher in the squids, and highest in the snails. High concentrations of ChE are found also in the muscles. Squid ganglion has the richest content of any invertebrate tissue (BOELL & NACHMANSOHN, 1940). ChE is lacking in the purple cyst of *Murex* (JULLIEN, 1939; JULLIEN & BONNET, 1941); from this animal ERSFAMER and DORDONI (1946) have isolated a new choline derivative (murexine) with a high ACh action which is not acted upon by ChE. Surprisingly high ChE activity is

found in the dart sac of *Helix pomatia* (AUGUSTINSSON, 1946 a, c). The enzyme is present in the blood, muscles, and the nervous system of echinoderms. Tunicates have no ChE in the blood, but the muscles have an ACh-hydrolysing activity.

The results mentioned have been obtained, amongst others, by AMMON (1935, 1943), ARTEMOW and his co-workers (with MITROPOLITANSKAJA, 1938; with LURJE, 1941; MITROPOLITANSKAJA, 1941), BACQ (1935, 1936, 1937; with NACHMANSOHN, 1937; with OURY, 1937), CORTEGGIANI and SERFATY (1939), FREDERICQ (1937), HALPERN and CORTEGGIANI (1935), JULLIEN (1939, 1941; with BONNET, 1941; with VINCENT, 1938, 1941; with VINCENT, BOUCHET & VUILLET, 1938), KOSI-TOYANTZ (1936), KAKUSHKINA and LEVINA (1946), MARNAY and NACHMANSOHN (1937 c), NACHMANSOHN (1938 b, 1939 a), PENNOIT-DE COOMAN (1930; with VAN GRAMBERGEN, 1942), REZEK and HAAS (1942), and by VINCENT and JULLIEN (1938, 1939; cf. VINCENT, 1938). These authors have viewed the problem from a physiological point. AUGUSTINSSON (1946 a, b, c) has determined the ChE content of various invertebrates chiefly from a chemical standpoint in order to find out more details about the specificity of the enzyme (p. 21). These studies have now been extended, the results of which are found in Part III.

h) *Bacteria*, in all probability, do not contain ChE according to BERNOULLI and BLOCH (1944). In contradistinction to this, SCHALLER (1942 a) found that the type I pneumococcus hydrolyses ACh; a low hydrolytic activity was also found for some bacteria by DE PRAT (1945) and VINCENT and DE PRAT (1945 b).

i) *Cholinesterase at different stages of development*. The investigations of the ChE activity at different stages of development have added much to our knowledge of the significance of the system ChE-ACh. It has been demonstrated in many ways that a relation exists between ChE concentration and function during embryonic development. The concentration is high at the time when nerve impulses begin to be transmitted.

In the muscle and the liver of chick embryos, the ChE concentration increases rapidly to high values during incubation, in the brain the concentration reaches high values before and after hatching (AMMON & SCHÜTTE, 1935; NACHMANSOHN, 1938 a, c, 1939 a, c; TORDA, 1938; DOMINI, 1938). THOMAS and NACHMANSOHN (1938) determined the ChE activity of the hearts of chick embryos. NACHMANSOHN (1940 a) showed that the concentration of ChE is high in the spinal cord at a very early age of the sheep foetus, but low at that time in the different brain centres. The ChE concentration is higher in the skeletal muscle of rabbit foetuses than in that of the adult animals (TORDA, 1938; LEIBSON, 1939 a; cf. RJABINOWSKAJA, 1940 a; KAKUSHKINA & ARKHIPOVA, 1941). SHAMARINA (1939) studied the ChE content in the embryonic auricles of various mammals and YOUNGSTROM (1941) determined the enzyme concentration during the development of the human foetus (central nervous system, skeletal muscles, liver). In the central nervous system the concentration runs parallel with the functional development (cf. NACHMANSOHN, 1938 h; SAWYER, 1942; BOELL & SHEN, 1944). Studying the respiration and cytochrome-oxidase activity of *Amblystoma*, BOELL (1945 b) has demonstrated that ChE and cytochrome oxidase appear at different times and at dissimilar rates during development. There appears to be a close relationship between the increase in ChE and ACh content and the maturation of the neural units in the developing chick retina (LINDEMAN, 1947).

The relationship between ChE and the development of behaviour in Amphibia has been studied by YOUNGSTROM (1938), ARTEMOW (1941), and SAWYER (1940, 1942, 1943 a, b) who also studied ChE during the development of *Fundulus* (a carp) (1944). TAHMISIAN (1943) investigated ChE in developing grasshopper eggs. The ChE activity of developing sea-urchin eggs has been determined by the present author and will be reported in Chapter VI (p. 71).

3. Correlation between Cholinesterase Activity and Various Pathological Conditions

The ChE concentration of the blood has been widely studied in order to correlate the enzyme activity with various pathological conditions. If such a relationship had been found it might have proved very useful both for diagnosis, as well as helping to elucidate the nature of many diseases. The mass of data obtained, however, has not allowed of any conclusions as to the relation between ChE activity and the diseases investigated. Moreover the marked variations of the activity of normal individuals make it difficult to explain the results and many determinations have been at variance with the expected findings. Therefore it must be pointed out that the data obtained do not seem to have any diagnostic value (cf. the review by AMMON & CHYTREK, 1939). They may possibly help in the search for a general factor governing the distribution of ChE (see Chapter XIII).

Investigations carried out with a great number of patients with various diseases have been described by ANTROPOL *et al.* (1937, 1938), McGEORGE (1937), MILHORAT (1938, 1941), CROXATTO and HUIDOBRO (1939), HUIDOBRO and CROXATTO (1939), McARDLE (1940), MENGOLI (1940), PIROLI (1941 b), FABER (1941, 1943 a), BUTT *et al.* (1942), RUMMA and SIBUL (1943), DE PRAT (1945), ARON and HERSCHBERG (1946), CRISTOL *et al.* (1946), LABORIT and MORAND (1946 a), VITTOZ (1946), and others.

Reduced serum ChE values have been obtained in the cases of liver disease (see also VINCENT & DE PRAT, 1942; SCHALLER, 1942 b; FIESSINGER *et al.*, 1944; VINCENT *et al.*, 1944; PASSOUANT *et al.*, 1945; KASWIN, 1945; RIMBAUD *et al.*, 1946). FABER (1941, 1943 a, b) has found a connection between the serum albumin concentration and the amount of ChE in serum and assumed that both the serum ChE and the albumin originated from the liver. BRAUER (1945), BRAUER and ROOT (1946, 1947), SAWYER and EVERETT (1947), and WESCOE *et al.* (1947) also found that the liver is the primary site of synthesis of the non-specific serum ChE (cf. STEENSHOLT & VENNDT, 1945; ELLIS, SANDERS & BODANSKY, 1947).

Low ChE values in the blood have been found in patients with anaemia, tuberculosis, carcinoma, dermatosis, uraemia, shock, etc., high values in hyperthyroidism, bronchial asthma, diabetes, and certain other diseases (see, amongst others, SCOZ & CATTANEO, 1937; CATTANEO, 1937; DIKSHIT & MAHAL, 1937; VEREBÉLY, 1937; JONES & STADIE, 1939; ALBUS, 1939; LACKEY & SLAUGHTER, 1939; SABINE, 1940; YOUNGSTROM *et al.*, 1941; RENTZ, 1941; KUCHINSKIĭ, 1941, 1942; BRÜCKE *et al.*, 1941; PIROLI, 1941 b; VINCENT & DE PRAT, 1942; WERLE & STÜTTGEN, 1942; KAKUSHKINA & TATARKO, 1942, 1947; WEBER, 1942; VILLASANTE, 1942; LANGEMANN *et al.*, 1943; FROMMEL, THALHEIMER, HERSCHBERG & PIQUET, 1943; HUIDOBRO *et al.*, 1943; RADOS, 1943; DE MICHELE, 1944; LANGEMANN, 1944 b; HERSCHBERG *et al.*, 1944 a; EPSTEIN *et al.*, 1944; SCHACHTER, 1945; VINCENT, BRYGOO & DE PRAT, 1945; PASSOUANT *et al.*, 1945; DAVIS, 1946; HEIM, 1946; HEIM & RUETE, 1946; BINET & BURSTEIN, 1946; VINCENT & BRYGOO, 1946; VINCENT & MALBEC, 1947; FROMMEL & PIQUET, 1947; STÜTTGEN, 1947). Much attention has been directed to a supposed relationship between serum ChE and blood pressure (ZINNITZ, 1940; ZINNITZ & RENTZ, 1940; PIROLI, 1941 a; cf. LONGO & SORRENTINO, 1940; STROPENT & BATTEZZATI, 1942; LIBBRECHT, 1945). Other investigations have stated that such a relationship does not exist (HALL & LUCAS, 1937 a; HUIDOBRO & CROXATTO, 1939; DOLES, 1940; BRÜCKE *et al.*, 1941; WERLE & STÜTTGEN, 1942; RIECHERT & FRISCH, 1942; SCHALLER, 1942 b; cf. also GOVAERTS *et al.*, 1931; HALL & ETTINGER, 1937; GINSBERG *et al.*, 1937; SARKAR *et al.*, 1942). Burning and freezing the muscles resulted in a reduction (FROMMEL & PIQUET, 1945, 1946) or raising of the serum-ChE activity (SCHÜRM-

MELFEDER, 1946, 1947 b). WILLIAMS (1944) found a definite biological difference between the "feminine" male homosexuals and all other individuals, in that there was an absence of the normal reduction in serum ChE following the subcutaneous administration of prostigmine.

According to SCHALLER (1942 b) the variations of ChE in erythrocytes and plasma are often due to a shift of the enzyme between the erythrocytes and the plasma; the enzyme is supposed to be formed in the red blood cells. As regards the quotient K/Ca in serum and the serum ChE activity, ARISI (1942) did not find any connection. This was not confirmed by RUMMA and SIRBU (1943), according to whom high values of K/Ca corresponded to low values of enzyme activity.

Besides these investigations, interest has been directed towards *myasthenia gravis* and *myotonia congenita*. The first disease is characterised by great muscle weakness and fatiguability and it has been known since 1934, that physostigmine or prostigmine can be used with good results in the treatment of myasthenia. As these two agents inhibit ChE specifically, it is logical to suppose that this disease may be associated with an imbalance between the rate of liberation of ACh at the myoneural junction and the rate of its destruction by ChE present locally, that is, deficiency of ACh brought about by increased ChE activity. However, evidence indicating that such a state of affairs actually exists is still lacking. Thus direct determinations of the serum ChE have shown, in most cases, normal values (McGEORGE, 1937; HALL & LUCAS, 1937 a; CORRILL & ENNOR, 1937; MILHORAT, 1938, 1941; FREUDENBERG & REDLICH, 1938; RUSSEL *et al.*, 1938; PONCHER & WADE, 1939; ODOM *et al.*, 1943; JUCKER, 1943; STONER & WILSON, 1943; WILSON & STONER, 1944; PASSOUANT *et al.*, 1946; cf. THOMPSON & TICE, 1941). Some cases have given high values (HICKS & MACKAY, 1936, 1938; GOODMAN *et al.*, 1939; ASK-UPMARK, 1939), whilst others have presented low ones (STEDMAN & RUSSELL, 1937; PICHLER, 1937; STOERK & MORFETH, 1944). Normal values for serum ChE, however, do not signify that the concentration of the enzyme at the neuromuscular junction is necessarily normal, on the contrary, some authors presume that ChE is abnormally elevated at the motor end-plates. No significant differences were found between the ChE activity of muscle from myasthenic patients and that from control subjects (JONES & STADIE, 1939; GOODMAN *et al.*, 1939). Other explanations of myasthenia gravis have been suggested (review: HOAGLAND, 1946). — Myotonia congenita, which is in complete contrast to myasthenia gravis, is characterised by muscular hypertonicity and slowness of relaxation. It has been surmised that this condition is related to an excess of ACh at the neuromuscular junction brought about by decreased ChE activity. Low serum ChE values in myotonia are reported by RUSSELL and STEDMAN (1936), and by HICKS and MACKAY (1938); also the myotonic muscle was found to have a low ChE level (MINZ, 1945; MINZ & PASSOUANT, 1945). PONCHER and WADE (1939) have obtained normal values for the serum ChE. The most effective therapeutic agent in myotonia is quinine, the relationship of which to ACh and ChE is not yet fully understood (see Chapter XI). — Other muscular (or nervous; cf. below) disorders have also been studied as regards their ChE activity (see ALTENBURGER, 1937; PICHLER, 1937; DIKSHT & MAHAL, 1937; MILHORAT, 1938; YOKOTI, 1939; AIL, 1940; FABER, 1941; ODOM *et al.*, 1943; VILLASANTE *et al.*, 1943; KASWIN, 1945).

A lowered activity of serum ChE has been found in disorders marked by reduced responsiveness (catatonic stupor, schizophrenia, epilepsy) and high values in anxiety states (JONES & TOD, 1937; TOD & JONES, 1937; JONES & STADIE, 1939; LONGO & COLACIURI, 1940; cf. BIRKHÄUSER, 1941 a, b; RICHTER & LEE, 1942; BUTT *et al.*, 1942). The lowered ChE activity in schizophrenia rises significantly after insulin treatment (RANDALL & JELLINEK, 1939; RANDALL, 1940).

At this juncture a short account of the investigations carried out on the connection between ChE activity and aneurine (and beri-beri respectively) appears germane. Aneurine inhibits ChE (GLICK & ANTROPOL, 1939 a, b; SÜLLMANN & BIRKHÄUSER, 1939; BRÜCKE & SARKANDER, 1940; BRÜCKE *et al.*, 1941; NITZESOU & TEODORU, 1941; AMMON, 1943; EGAÑA, 1946); some authors, however, have denied this (BYER & HARPUDER, 1940; SAVIANO, 1942; ROCA & LLAMAS, 1943; cf. LISSAK *et al.*, 1943). Also acetylaneurine (cf. p. 17, Table 2) is said to be a competitive inhibitor for ChE. Cocarboxylase, which is not hydrolysed by ChE,

has the weakest effect of the three aneurine derivatives (GLICK & ANTROPOL, 1939 a; cf. EGAÑA, 1946; ROCA & LLAMAS, 1943).

If vitamine B₁ inhibits ChE *in vivo*, it would be expected that the ChE concentration would be increased in B₁-deficient animals. Indeed, ANTROPOL *et al.* (1939 b), LISSÁK *et al.* (1943), and FROMENT & KASWIN (1945) obtained increased ChE activity of blood serum, striated and intestinal muscles of beri-beri animals (cf. BRÜCKE & SARKANDER, 1940). In contradistinction to this ZELLER and BIRKHÄUSER (1940) have shown that the ChE concentration in rat liver is definitely diminished in animals suffering from beri-beri and PIGHINI (1939 c) found lower values than normal in brain, spinal cord, and sciatic nerves of beri-beri pigeons (cf. EGAÑA, 1946).

CHAPTER II

THE CHEMISTRY OF CHOLINESTERASE

A. SPECIFICITY OF CHOLINESTERASE

1. Earlier Investigations

According to recent findings we know that the ACh-hydrolysing factor cannot be ascribed to a single enzyme. Previously, when ChE was supposed to be a single entity, several investigations were undertaken in order to determine the relationship between the structure of various substrates and the action of ChE (from serum) upon them. These earlier investigations on the specificity of ChE were carried out by SIMONART (1931, 1933) and latterly mainly by STEDMAN and his co-workers (STEDMAN, STEDMAN & EASSON, 1932; STEDMAN, STEDMAN & WHITE, 1933; EASSON & STEDMAN, 1936, 1937), KAHANE and LÉVY (1936 c, 1937 b), ROEPKE (1937), ROEPKE and WELCH (1936), and especially by GLICK (1938 c, 1939 a, 1941 b). SIMONART pointed out that serum contains a non-specific "acetylcholinase". STEDMAN's group found evidence that serum ChE is an enzyme distinct from lipase and simple esterase. KAHANE and LÉVY investigated the hydrolysis of some choline esters in connection with the effect of physostigmine in sensitizing tissues (serum, muscles of leech and frog) to the pharmacological action of these esters. A more systematic study was made by GLICK. He measured both the enzymic and non-enzymic hydrolysis of a variety of choline esters with various structures of the acid component and the hydrocarbon portion of the alcohol component. Table 2 summarises some substrates studied in relation to blood serum which, as we know, contains in most cases a non-specific ChE. *N.B.* ChE refers to *serum ChE*.

The enzymic hydrolysis increases with lengthening of the acid component of the choline esters from 2 to 4 carbon atoms (I—IV); the non-enzymic hydrolysis is decreased with this lengthening. After the butyryl compound, the length of the acid chain (VI) results in a decrease of the enzymic hydrolysis. This effect no longer holds in the case of chains long enough to render the compounds water-insoluble (VII). The hydrolysis decreases when branching alkyl groups are introduced in the acid component (V). The choline esters of dicarboxylic acids are

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split relatively slowly (XI). The pyruvoyl-Ch (VIII) is attacked by the serum ChE which also hydrolyses the benzoic-acid ester (IX); the *p*-nitro, *p*-amino, and *p*-methyl derivatives of benzoylcholine are not split (HANDLEY, 1946). Carbaminoyl-Ch (X), as well as substituted ones (e.g., dibutoline, PETERSON & PETERSON, 1945), are not affected by the enzyme. The same holds for some recently prepared choline esters (furoyl-, thenoyl-, nicotinyl-) (CARR & BELL, 1947). In the case of ester analogues of choline esters where the quaternary ammonium radical is included in a ring structure (XII), hydrolysis in a manner similar to that found with the corresponding straight chain esters takes place though not so rapidly. Inorganic esters of choline (XIII, XIV) are not enzymically hydrolysed by serum ChE. Introduction of an α -methyl group into the choline portion (XV) results in a reduction in activity which is more pronounced when the methyl is placed in the β -position (XVI). Introduction of a CH_2 group between the substituted amino radical and the ester link, as in acetyl- γ -homo-Ch (XVII), decreases the enzymic effect. When two methyl groups are added to the centre carbon atom in this γ -homo-Ch, little change is produced. Esters of thiocholine (XVIII) are more rapidly split by ChE than their oxygen analogues. ChE hydrolyses substances where the nitrogen atom of choline is replaced by phosphorus or arsenic (XIX) at practically the same rate as ACh. Acetylcolamin (XX), where the amino radical is *ur*-substituted, is unaffected by ChE. In contradistinction to KAHANE and LÉVY, GLICK found that the ethyl ester of betaine (XXI) is not acted upon enzymically by serum.

Serum ChE seems also to have a stereochemical specificity. Thus GLICK (1938 c) tested the optical isomers of acetyl- β -methyl-Ch (XV) and found that only the *D*-compound was enzymically attacked and not the *L*-ester.

Essentially different from the choline esters is acetylcholine (XXII), synthesised for the first time by KUHN, WIELAND and HUEBSCHMANN (1939) (cf. p. 5). It is most probably the same factor which in blood serum splits ACh and acetylcholine as demonstrated by AUGUSTINSSON (1944). Already MASSART and DUFAIT (1939 b, 1940 a), SÜLLMAN and BIRKHÄUSER (1959), and RIECHERT (1940) had found that serum, brain, and certain other tissues split acetylcholine. AUGUSTINSSON (1944) announced that the blood of *Helix* hydrolyses this ester at a high rate. RIECHERT and WIELAND (1941) who have reported hydrolysis of acetylcholine by leech extracts, found that these extracts also hydrolyse benzoylcholine. They supposed that it was the same enzyme hydrolysing both ACh and the two choline esters. The enzymic hydrolysis of acetylcholine (ACh) will be discussed more fully in Part III.

2. Different Types of Cholinesterase

Until the year 1940, uncertainty prevailed as to whether the hydrolysis of ACh was catalysed by a specific enzyme or not. When in 1932 STEDMAN and his co-workers proposed the name "choline-esterase" for the choline-ester hydrolysing enzyme in blood serum (horse), it was impossible to decide whether there was more than one enzyme responsible for the hydrolysis of choline esters, methyl butyrate, and tributyrin. The following year the same authors (STEDMAN, STEDMAN & WHITE, 1933; WHITE, 1933; EASSON & STEDMAN, 1937) announced that many sera contained two enzymes at least, one ChE, the other an ordinary esterase. The specificity of the two was not absolute. In addition, the existence of a third enzyme, a lipase, was assumed.

In 1935, SHAW demonstrated two esterases in serum one of which was inhibited by physostigmine. VAHLQUIST (1935) was the first who definitely pointed to the fact that ChE in human blood plasma is not specific against choline esters. Two years later, HALL and LUCAS (1937 b) investigated the hydrolysis of ACh, methyl butyrate, and tributyrin by sera from 77 various animals and emphasized that it was impossible to draw definite conclusions regarding the specificity of the three different enzymes present. According to FRANCIOSI (1937) choline phosphatase, hydrolysing the choline ester of glycerophosphoric acid is not identical with ChE. Phosphorylcholine, studied by BEZNAK and CHAIN (1937), is most probably not hydrolysed by the serum ChE. The serum phosphatase is not identical with serum ChE (CRISTOL, PASSOUANT, BENEZECH & DUTARTE, 1945). This, most probably,

holds also for the other azolesterases of serum, the morphine esterases (WRIGHT, 1942), the tropine esterases (GLICK & GLAUBACH, 1941; GLICK, GLAUBACH & MOORE, 1942; LÉVY & MICHEL, 1945; BLASCHKO, CHOU & WAJDA, 1947 a), and the procaine esterase (KISCH, KOSTER & STRAUSS, 1943).

It was in 1939, so far as the author knows, that GILMAN, CARLSON and GOODMAN first spoke about a "specific" and a "non-specific" ChE. They found that the esterase of some rat tissues could be almost completely inhibited by minute amounts of physostigmine; this was the "specific" ChE. However, the esterase of other rat tissues (kidney, skeletal muscle) was little affected even in the presence of high concentrations of this alkaloid; this was the "non-specific" ChE. Apart from this short communication before 1940, little had been said about the specificity of ChE in tissues others than blood.

It had long been known that the erythrocytes have an ACh-hydrolysing effect. The next question that arose was whether the erythrocyte ChE and the serum ChE were identical. In 1940 this problem was investigated experimentally for the first time on human blood by ALLES and HAWES (1940, 1944) (HAWES & ALLES, 1941; cf. the controversy in *Science* between these authors and MENDEL's group, clarified by GLICK, 1945). These authors pointed out that the enzyme of serum and that of erythrocytes are distinct. This conclusion was based on the difference in the enzymic properties of the enzymes regarding activity-pH, activity-NaCl, and activity-substrate concentration relationships. ALLES and HAWES observed that excess of ACh depressed the activity of the cell ChE which was not the case with the serum ChE. Acetyl- β -methylcholine was split by the cells at the same rate as ACh, the serum, on the other hand, hydrolysed the β -methyl derivate only slightly. These observations have more recently been confirmed and considerably extended by various groups of investigators, but, as GLICK claims, "ALLES and HAWES deserve the priority for the initial discovery".

RICHTER and CROFT (1942) have shown that the hydrolysis of methyl butyrate and tributyrin by human serum is brought about chiefly by ChE, thus confirming the earlier observation of VAHLQUIST (1935). The ChE of the erythrocytes is said to be different from that of serum in having an absolute specificity against choline esters. Moreover, the two enzymes behave differently in relation to certain substances.

MENDEL and RUDNEY (1943 a) examined highly purified enzyme preparations (cf. MENDEL & MUNDELL, 1943; MENDEL, 1943; STRELLITZ, 1944) and found that ChE in blood serum and certain animal tissues is a non-specific ChE hydrolysing not only esters of choline but also a variety of non-choline esters. In addition a specific or true ChE exists, acting exclusively on certain choline esters. MENDEL and RUDNEY have called the non-specific enzyme a "pseudo-cholin-esterase" which has been considered "an unfortunate designation" (LAUBENFELS, 1943; cf. MENDEL & RUDNEY, 1944 a), inadvisable to accept (ALLES & HAWES, 1944; cf. MENDEL & RUDNEY, 1944 b), and which "ought to be dropped from the scientific literature as soon as possible" (GLICK, 1945). A similar observation to that of ALLES and HAWES was that the non-specific ChE of blood serum exhibits its maximum activity at high ACh concentrations ($> 2 \times 10^{-2}$

M) and displays decreasing activity with diminishing substrate concentrations; the specific ChE of brain and red blood cells, on the other hand, exhibits its maximum activity at low ACh concentrations ($< 2 \times 10^{-4}$ M) and displays increasing inhibition with rising substrate concentrations.

Since the above work the Canadian group has extended these observations. Thus MENDEL, MUNDELL and RUDNEY (1943) reported that acetyl- β -methylcholine is hydrolysed only by the "true ChE" and not by the "pseudo-ChE", whereas benzoylcholine is split only by the "pseudo-ChE", and not by the "true ChE" (cf. VINCENT, 1946 b). Using this method, MENDEL and RUDNEY (1943 b) found that brain tissue of all vertebrates contains only "true ChE". As regards other organs no general statement concerning the type of ChE could be made. "True ChE" seems to be located in the erythrocytes of most blood species, in some cases (cats, birds) in the plasma. "Pseudo-ChE" is lacking in some species (ox, sheep) (MENDEL, MUNDELL & RUDNEY, 1943), and, according to GUNTER (1946), it seems to be absent on the whole from the tissues of ruminants. The results of HAWKINS and GUNTER (1946) have given evidence that "pseudo-ChE" plays no essential part in the hydrolysis of ACh *in vivo*. In this respect it is interesting to note that SAWYER (1945) has found an esterase — a "benzoylcholine esterase" — in the liver of guinea pigs (also in that of rabbits) capable of splitting benzoylcholine, but not engaged in the hydrolysis of ACh. The existence of such an esterase will be discussed more fully in Part III. MENDEL and his co-workers found that the shape of the curve relating initial velocity and substrate concentration is changed by the addition of salt (KCl) or protamine to the medium containing "true ChE" (MENDEL & RUDNEY, 1945; HAWKINS & MENDEL, 1946) (see Chapter IX).

The problem of the specificity of ChE was also studied in Basle about the same time. ZELLER and BISSEGGER (1943) (cf. ZELLER, 1944) presumed the existence of two types of ChE: ChE of brain and erythrocytes constituting the "e-type" which is different from the "s-type" or ChE of blood serum. The two types are different in their behaviour to certain substances (see Table 3). It was also shown that the activity of the brain ChE is depressed by excess of substrate, a property similar to that of the erythrocyte ChE, observed by ALLES and HAWES.

LANGEMANN (1944 a) studied the ChE types in various human tissues, and GLASSON (1944, 1945) found that human erythrocytes contain one specific ChE and one "aliesterase" and serum contains one non-specific ChE. The different enzymes seem also to be present in the cerebrospinal fluid (GLASSON & MUTRUX, 1946).

AUGUSTINSSON (1944, 1945) has shown that a true chemically established difference exists between the ACh-hydrolysing enzymes in horse blood serum and erythrocytes. Electrophoretic investigations showed that a large difference exists between the rates of migration of the two enzymes in the electric field. Furthermore, erythro-

TABLE 3. *Characteristics of the Two Acetylcholine-Hydrolysing Enzymes*

| Characteristics | ChE I | ChE II | References |
|--|--|---|------------------------------------|
| Nomenclature | Specific ChE
"True ChE"
"c-Type"
Cholinesterase | Non-specific ChE
"Pseudo-ChE"
"s-Type"
Unspecified ester-
ase | 211, 227
398
685
459, 462 |
| General distribution | Erythrocytes,
nerve tissues,
thymus, etc. | Blood serum,
pancreas, glands,
etc. | See Table 1 |
| Optimum pH | 7.5-8.0 | 8.5 | 10; cf. 495 |
| Isoelectric point | 4.65-4.70
(erythrocytes) | 4.36
(serum) | 40 |
| Permanence at different pH . | less | more | 40, 41 |
| Inhibition by excess of sub-
strate | + | - | 10, 398, 685, 459,
44, 106 |
| Activation by NaCl | + | - | 10; cf. 225, 402 |
| Hydrolysis of: | | | |
| tributyrin | - | + | 618, 512, 398, 459 |
| acetyl- β -methyl-Ch | + | - | 10, 395, 459 |
| benzoyl-Ch | - | + | 395, 459 |
| phenylacetyl-Ch | - | + | 530 |
| atrolactyl-Ch | - | + | 71 |
| Inhibition by: | | | |
| quaternary N-bases | ++ | + | 543; cf. 143 |
| tertiary N-bases | + | ++ | 543 |
| dimethylcarbamate of 2-
hydroxy-5-phenyl-benzyl-
trimethyl-ammonium bro-
mide | - | + | 252 |
| caffeine | ++ | - | 685, 462, 143 |
| percaïne, irgamid | + | ++ | 685, 143 |
| priscoï | ++ | + | 561 |
| prinine | + | ++ | 561 |
| quinine | + | ++ | 462 |
| intocostrin (curare) | + | ++ | 249 |
| paludrine | + | ++ | 72 |
| di-isopropyl fluorophosphate
(DFP) | + | ++ | 254 |

cyte ChE seems considerably more sensitive to pH than serum ChE.

Latterly AUGUSTINSSON has extended his studies on the specificity of ChE. In an investigation on ChE in different marine invertebrates (1946 a, b), the ACh hydrolysing effect was compared with the hydrolysis of acetyl- β -methylcholine and benzoylcholine, whose use had been suggested in order to estimate the specificity of ChE (MENDEL, MUNDELL & RUDNEY, 1943). It was shown that some species hardly split either of the two substrates and still others split acetyl- β -methylcholine at a higher rate than ACh (cf. RICHARDS JR. & CUTKOMP, 1945; TOBIAS *et al.*, 1946). A more detailed study has been performed with *Helix pomatia* (AUGUSTINSSON, 1946 c). These studies have now been extended and the results are found in Part III.

NACHMANSOHN and ROTHENBERG (1945) determined that the esterase in human serum is "unspecified", and that in the erythrocytes it is specific for ACh. The esterase in all nervous tissues, motor and mixed nerves as well as sensory nerves (BULLOCK, GRUNDFEST, NACHMANSOHN and ROTHENBERG, 1947) is either exclusively or predominantly ChE, so also in striated and heart muscle both free of nerve endings (also, ROTHENBERG & NACHMANSOHN, 1945). NACHMANSOHN and SCHNEEMANN (1945) compared the effect of some drugs on ChE. In phenylacetylcholine, ROTHENBERG (1945) found a compound which is completely resistant to ChE, whereas other esterases are able to split it.

The activity-substrate concentration relationship of ChE has also been studied by CASIER and DELAUNOIS (1946).

A comparison of the two types of ACh-hydrolysing enzymes is found in Table 3.

B. SYNTHESISING ACTION OF CHOLINESTERASE

As early as 1926 ABDERHALDEN and PAFFRATH found during experiments with the small intestine of pig that the ACh-hydrolysing enzyme has a synthesising effect strongest at pH 6, as opposed to a hydrolysing one. These results have been later confirmed in experiments with pig serum (SHAW, 1935), embryonic extracts (AMMON & KWIATKOWSKI, 1934), leech extracts, and human serum (KWIATKOWSKI, 1936). In respiration experiments with brain slices, QUASTEL, TENNENBAUM and WHEATLEY (1936) obtained a substance which was supposed to be a choline ester; they did not find any connection between the ChE concentration of various tissues and the ability of synthesising choline esters. In all these instances, the synthesis took place aerobically.

It is certain that ACh is synthesised during the electric stimulation, for example of the superior cervical ganglion, but it is as yet not clear whether the ACh formed is identical with that liberated at the nerve endings or synapses. This synthesis at the cervical ganglion is not inhibited by physostigmine; therefore it is doubtful whether this process is catalysed by ChE. The formation of ACh is increased by adding glucose, lactic acid, acetoacetic acid, and pyruvic acid. These substances of carbohydrate metabolism have no influence on the ChE activity, but they are sources of the acetyl group needed for the synthesis of ACh. The phospholipids are the origin of choline.

NACHMANSOHN and MACHADO (1943) have shown that ACh is also formed anaerobically. They have extracted from brain a new enzyme, *choline acetylase*, which, under strictly anaerobic conditions in the presence of adenosine triphosphate and K^+ , forms ACh in cell-free solution. Physostigmine and fluoride must be present in order to inhibit the action of ChE and adenosine triphosphatase respectively. The preparation of the enzyme has been described. The enzyme seems to be present also in striated and cardiac muscle. These findings by NACHMANSOHN and his co-workers have been in part confirmed by other authors.

C. DETERMINATION OF CHOLINESTERASE ACTIVITY

In the quantitative determination of ChE activity, the rate of hydrolysis of added ACh is measured. Formerly biological methods were chiefly used, but today chemical ones are more in favour. In the biological methods high enzyme- and low substrate-concentrations are employed, whereas in the chemical ones the substrate is in excess

and the enzyme in dilute solution. Because of these differences, comparable results are not obtained with the two methods (cf. MAHAL, 1938).

1. Biological Methods

The evanescence of ACh is followed pharmacologically by measuring its action on isolated intestine (ABDERHALDEN & PAFFRATH, 1926; AHLMARK & KORNERUP, 1939), heart (LOEWI & NAVRATIL, 1926; PLATTNER & HINTNER, 1930; HELLAUER, 1939), frog muscle (SCHEINER, 1939; CRIVETZ, 1945), leech muscle (MINZ, 1932), frog rectus (MARTINI & TORDA, 1937), or by measuring the blood pressure (DANTELOPOLU & POPESCO, 1946).

2. Chemical Methods

In estimating ChE chemically, the acetic acid liberated by the hydrolysis of ACh is determined. Such a method is used almost exclusively today.

a) Titration Methods

The liberated acetic acid is titrated with an alkaline solution of known concentration.

(1) *Indicator methods.* Various indicators have been recommended, e.g., phenolphthalein, phenol red, cresol red, or bromthymol blue. The method has been described by RENSCHAW and BACON (1926), STEDMAN, STEDMAN and EASSON (1932), WHITE (1933), VAHLQUIST (1935), HALL and LUCAS (1937 a), ROEPKE (1937), PIGHINI (1939 a), EBERHARD and SILVERMAN (1939), BOVET and SANTE-NOISE (1941), and others. In connection with enzymic-histochemical investigations, a micro-titration method for assay of ChE has been described by GLICK (1937 a, 1938 a, b). GLICK's method has been modified by SAWYER (1943 a). In using dyes as indicators, it is often very difficult to observe a clearly defined colour change. In addition, many other troublesome precautions must be observed, not the least being the possible influence of the indicator on the enzyme (cf. BERNHEIM & BERNHEIM, 1936; LONGO, SORRENTINO & COLACIURI, 1940; PIGHINI, 1941; SACK & ZELLER, 1943).

(2) *Electrometric methods.* A more convenient method than using indicators is to titrate electrometrically. It is advisable to use a quinhydrone electrode (SCOZ & CATTANEO, 1937; BARBOUR & DICKERSON, 1939), glass electrode (GLICK, 1937 a; ALLES & HAWES, 1940; SANZ, 1944; SCHÜMMELFEDER, 1947 a), or antimony electrode (DELAUNOIS & CASIER, 1946). Electrometric titration, details not given, was also employed by GILMAN, CARLSON and GOODMAN (1939).

b) Manometric Methods

(1) *WARBURG method.* The WARBURG manometric method is the most convenient for following the hydrolysis of ACh, it is also the most suitable for use in comparative and serial experiments. AMMON (1933) was the first to use this method in assaying ChE activity. The basis of the method is the manometric estimation of the volume of CO_2 evolved from a bicarbonate-containing system buffered against CO_2 by the acetic acid formed in the hydrolysis of ACh. This method

is now used almost exclusively and will be described in detail in Chapter III.

(2) *BARCROFT differential method* has been employed by STEDMAN and STEDMAN (1935 a).

(3) *VAN SLYKE method* has been used by RINKEL and PIJOAN (1938); it was employed in a modified form by FRIEND and KRAYE (1941).

(4) *Microchemical gasometric method*. The principle of the Cartesian diver has been successfully applied to the study of the ChE activity by LINDERSTRÖM-LANG and GLICK (1938). This microchemical gasometric technique has also been used by BOELL and SHEN (1944).

c) Other Methods

A step-photometric method, based on the ferric-chloride reaction of acetic acid, has been described by ARDON and UVNÄS (1937). This method is one of those used by ALI (1940) and by PIROLI (1941 a, b). Another photometric method, using *m*-nitrophenol as indicator has been evoked by CROXATTO, CROXATTO and HUDONRO (1939).

An opalescence method was introduced by GAL (1940).

3. Methods Proposed for Distinguishing between Specific and Non-Specific Cholinesterase

After evidence had been presented that two types of choline-ester splitting enzymes exist, various methods were proposed in order to distinguish between them. Thus MENDEL, MUNDELL and RUDNEY (1943) based a method, now frequently used, on the observation that acetyl- β -methylcholine is split only by the specific ChE and not by the non-specific ChE, whereas benzoylcholine is hydrolysed only by the non-specific and not by the specific ChE. More recently other authors have used other choline esters; ROTHENBERG (1945), for instance, employed phenylacetylcholine which is split only by the non-specific ChE.

A biological method has been proposed by DENYS and LÉVY (1947 a), using frog muscle and various choline esters.

D. UNITS USED IN EXPRESSING CHOLINESTERASE ACTIVITY

(1) NACHMANSOHN's *Q* and *A*. A very useful unit of the ChE activity is NACHMANSOHN's *Q* (NACHMANSOHN & LEDERER, 1939 b). This is mg. ACh hydrolysed in 60 min. by 100 mg. tissue at 20° C. In later investigations ROTHENBERG and NACHMANSOHN (1947) have used the unit *A* or mg. ACh hydrolysed in 60 min. by 1 mg. protein.

JONES and TOD (1935) assigned ml. CO₂ evolved per minute by the effect of serum. Otherwise, the ChE activity is expressed as the volume of CO₂ in μ l. evolved in 60 min., or sometimes as the quantity of ACh in μ mol. hydrolysed during the same time period.

(2) AUGUSTINSSON's *Cf* and *b*₃₀. *Cf* signifies the quantity of ACh in μ mol. destroyed in 60 min. by 1 mg. dry substance (AUGUSTINSSON, 1944). In this paper the ChE activity is symbolised by *b*₃₀ which represents the amount of CO₂ in μ l. evolved during 30 min. (Chapter III).

E. CHOLINESTERASE PREPARATIONS

Purified preparations of serum ChE have been obtained by STEDMAN and STEDMAN (1935 b) by fractional precipitation with ammonium sulphate. A similar method was used by McMEEKIN (1939) who tried also precipitation with lead acetate. GLICK, GLAUBACH and MOORE (1942), applying the electrophoretic method for the separation of serum proteins, found that ChE is associated with the α - and β -globulin fractions. According to FABER (1943 b) and AUGUSTINSSON (1944) ChE is precipitated by ammonium sulphate in the albumin fraction and a close relationship seems to exist between these two substances. Other investigations have also shown that the enzyme activity is limited to the albumin fraction.

Methods for purifying serum ChE are described also by AUGUSTINSSON (1944) and STRELITZ (1944). The principle of the first method is described in Chapter IV. BADER, SCHÜTZ and STACEY (1944, 1945) have isolated a crystalline serum mucoprotein with high ChE activity (cf. MENDEL, RUDNEY & STRELITZ, 1944).

The preparation of ChE of red blood cells may be performed by adsorbing the enzyme on infusorial earth (MENDEL & RUDNEY, 1943 a), or by extracting it with ammonia solution of pH 8.3 (MENTHA, SPRINZ & BARNARD, 1947). A further method is described by the present author in Chapter IV.

The first experiment with purified ChE of animal tissue (electric organ) was carried out by NACHMANSOHN and LEDERER (1939 a, b). Recently, ROTHENBERG and NACHMANSOHN (1947) have described a method for the purification of ChE from the electric organ of *Electrophorus* by fractional ammonium-sulphate precipitation. In the solutions obtained, 1 mg. of protein split 20 000 to 21 000 mg. of ACh per hour. MENDEL and MUNDELL (1943) have described a method of preparing "pseudo-ChE" from dog pancreas.

CHOWDHURY (1942) has purified ChE from snake venom by fractional precipitation with sodium sulphate and ammonium sulphate. Continuous purification with electrophoretic and adsorption methods gave practically "pure" ChE (CHOWDHURY, 1944), the isoelectric point of which was 5.55 (5.9) (CHOWDHURY, 1946). ChE of snake venom has also been studied in an isolated state by SARKAR, MAITRA and GHOSH (1942).

F. THE PROPERTIES OF CHOLINESTERASE

1. Chemical Nature of Cholinesterase

ChE is a protein. The isoelectric point has been determined by AUGUSTINSSON (1944) and fixed at pH 4.36 for serum ChE and pH 4.65—4.70 for erythrocyte ChE (horse) (cf. CHOWDHURY, 1942, 1946). The enzyme which is inactivated by dialysis may be reactivated by adding the dialysate (NACHMANSOHN & LEDERER, 1939 b) or metallic ions (MASSART & DUFAY, 1939 a, 1940 b, 1941 b; NACH-

MANSOHN, 1940 b). Primarily, bivalent ions show this effect. According to MASSART and DUFAYT, Ca^{2+} is the physiological activator of ChE. NACHMANSOHN and LEDERER (1939 a, b) have assumed that the ChE molecule (electric tissue) contains sulphydryl groups. They have shown that the enzyme is inhibited by those substances (e.g., maleic acid, oxidised glutathione) which are capable of transferring SH groups to SS groups. These results are at variance with those obtained in the present investigation performed with enzyme preparations from warm-blooded animals (see Chapters IX and XI).

BARNARD (1943, 1946) has assumed a possible haeme nature of ChE.

2. Factors Influencing the Kinetics of Cholinesterase

a) *Effect of concentration of enzyme.* All experiments carried out in order to find the relationship between the rate of ChE action and the concentration of the enzyme have shown that a direct ratio exists, within the rather wide variation of the latter. This relationship will be discussed more fully in Chapter VIII.

According to EASSON and STEDMAN (1936), each active ChE centre can hydrolyse 1 500 molecules ACh per sec. (the "turnover number"). The molar concentration of ChE centres in undiluted serum (horse) is 7×10^{-3} . The experiments of CLARK and RAVENTÓS (1938) made on the isolated frog auricle indicated that the ChE activity of the auricles is equal to that produced by a concentration of enzyme centres equal to 2×10^{-5} . STRAUS and GOLDSTEIN (1943) and GOLDSTEIN (1944) have estimated the molar concentration of ChE centres in 4.54 % serum (dog) to be less than 1.8×10^{-8} .

b) *Effect of concentration of substrate.* The study of the relationship between the activity and concentration of the substrate has been of great interest in the development of our knowledge of the ACh-hydrolysing factor. This relationship was one of the first clues in the separation of the two different types of ChE. Thus the specific ChE is said to be inhibited by excess of substrate, whilst the non-specific ChE is not (p. 20). Before research had progressed so far, several investigations were carried out in order to find out how the reaction velocity was influenced by the concentration of ACh. In these studies blood serum was used. A detailed account on the activity-substrate concentration relationship will be found in Chapter IX.

c) *Effect of concentration of hydrogen ions.* At pH 2, the non-specific ChE (horse serum) is completely destroyed; on the alkaline side the enzyme activity starts to decline at pH 11 (WERLE & UEBELMANN, 1938; AUGUSTINSSON, 1944; STRELITZ, 1944; MIHAILESCO, 1946). With the specific ChE (erythrocytes), preparatory experiments have shown that at pH 4.5 the enzyme is already completely destroyed (cf. p. 58 and AUGUSTINSSON, 1944). The stability at alkaline reactions also seems to be less than for the serum ChE. In this respect, the brain ChE (ox) is much the same as the erythrocyte ChE (AUGUSTINSSON, 1945).

The optimum pH of ChE has been determined by EASSON and STEDMAN (1936), BERNHEIM and BERNHEIM (1936), GLICK (1937 a,

1938 a), and WERLE and UEBELMANN (1938). The values were 8.0, 8.4, 8.5, and 8.0—8.5 respectively. All these experiments were carried out with the non-specific ChE (serum). For the specific ChE (erythrocytes), ALLES and HAWES (1940) obtained a somewhat lower value for the optimum pH, 7.5—8.0. Similar observations had previously been made in 1928 by PLATTNER *et al.*

d) *Effect of temperature.* ChE in tissue extracts is fairly stable, this applies particularly to glycerine extracts (GLICK, 1937 a; RIECHERT & SCHNARRENBARGER, 1942). For blood serum this has been shown by several authors (cf., however, VINCENT & BRYGOO, 1945). ZACHOWSKI and AMMON (1943) demonstrated that in blood kept in different ways the ChE activity increased with time. This was thought to be due to the slow passage of ChE from the red cells into the plasma. A 42 year-old sample of horse blood (haemolysed) collected aseptically and preserved in the dark at room temperature contained up to 85 per cent of its original ChE activity (KEILIN & WANG, 1947).

At about 56°C, the enzyme begins to be destroyed (LOEWI & NAVRATIL, 1926; PLATTNER & HINTNER, 1930; GLICK, 1939 b), at 70°C the activity is quite lost (ABDERHALDEN & PAFFRATH, 1926; KAHANE & LÉVY, 1936 a; WIECZOREK, 1937). Evaporation of blood and tissue extracts gives active dry powders (BERNHEIM & BERNHEIM, 1936); drying with acetone, however, destroys the enzyme (NACHMANSOHN & LEDERER, 1939 a). The freezing-drying process does not affect the ChE activity (ANFINSEN, LOWRY & HASTINGS, 1942; BOELL & SHEN, 1944; BOELL, 1945), shown also by the present author with sea-urchin eggs (p. 71).

The temperature coefficient of ACh hydrolysis by blood was determined by Plattner *et al.* (1928), VAHLQUIST (1935), ABDON and UVNÄS (1937), and GLICK (1939 b).

The critical increment (apparent energy of activation) was about 5 000 Cal. The optimum temperature of the ChE activity of serum and nerve tissues is 37—40° C (KAHANE & LÉVY, 1936 a; CLARK *et al.*, 1938; GLICK 1939 b; PIGHINI, 1939 a; GENUIT & LABENZ, 1941).

e) *Effect of light and other radiations.* As regards the visible spectrum nothing is known of the influence of its different components on ChE. The enzyme seems to be unaffected by ultraviolet light (cf., however, STÜTTGEN, 1947) and the light of fluorescence (ENGELHART & LOEWI, 1930). KWIATKOWSKI (1936) has reported that X-rays have no definite effect on ChE.

3. Activators

a) *Metallic ions.* In the first place, certain bivalent metallic ions act as activators on ChE. This has been shown by several groups of investigators (MASSART & DUFAYT, 1939 a; DUFAYT & MASSART, 1939; MENDEL, MUNDELL & STRELITZ, 1939, 1940; NACHMANSOHN, 1940 b; PUNT, 1942; FROMMEL *et al.*, 1943; SCOZ & DE MICHELE, 1944 b). The activating effects of Ca^{2+} , Mg^{2+} , and Mn^{2+} are well established, but there are different opinions regarding the amplitude of these effects. According to NACHMANSOHN, Mg^{2+} is more effective than Ca^{2+} . Sr^{2+} seems to activate weakly, Ba^{2+} and Cd^{2+}

have no effects at all. Cu^{2+} and Co^{2+} inhibit ChE action and Ni^{2+} is an activator or inhibitor, according to the concentration used.

Regarding the actions of monovalent ions (K^+ and Na^+) the disagreements have been considerable. They are resolved, however, if we own that ChE from various sources have been studied. Thus, NACHMANSOHN (1940 b) found that these ions activated the enzyme of *Torpedo* electric organ. GLICK (1941 c) observed an activation of rat serum ChE by K^+ and Na^+ , but this was not the case with horse serum. ALLES and HAWES (1940) claimed that the ChE of red blood cells but not that of serum (man) was activated by NaCl (cf. ROEPKE, 1937). In earlier papers, MENDEL's school (p. 20) reported an inhibiting action of K^+ , also found by FROMMEL *et al.* (1944). Recently, MENDEL and his co-workers (MENDEL & RUDNEY, 1945; HAWKINS & MENDEL, 1946) described the action of K^+ in more detail (cf. p. 97). The action of KCl and in some cases of LiCl has been studied in detail by the present author (Chapter IX).

b) *Organic substances.* KEESER (1938) reported an activation of serum ChE (man, horse) by glutathione, sympathol, and pilocarpine. NACHMANSOHN and LEDERER (1939 a, b) found such an activation only with reduced glutathione (and also cysteine). Regarding the actions of sympathol and pilocarpine other investigators could not confirm KEESER's results (see Table 4).

The ChE activity seems to be increased by glucose, hydrocyanic acid, and salicyl aldoxime (NACHMANSOHN & LEDERER, 1939 b), by ammonia (SEIDLITZ, 1939; FROMMEL *et al.*, 1944), and by neutral fats, palmitic acid, and oleic acid (FORBES, OUTHOUSE & LEACH, 1940). ARON, HERSCHBERG and FROMMEL (1944) have found that glycine, alanine, proline, and particularly arginine, lysine, and histidine potentiate the action of ChE both *in vitro* and *in vivo*.

An activator of ChE of unknown chemical composition has been found in impure preparations of kallikrein (WERLE & STÜTTGEN, 1942). Another substance augmenting the ChE activity of rat heart muscle was obtained by WRIGHT and MENDEL (1946).

GRANZNER (1939), RUBINO (1940), and FROMMEL, HERSCHBERG and PIQUET (1943) have obtained activation of serum ChE *in vivo* by vitamin C, but further investigations by other workers have not confirmed this (Table 4). In female rats the ChE activities of brain, serum, and liver seem to be reduced in E-avitaminosis (BLOCH, 1942). The activity may be regained by adding DL- α -tocopherol acetate to the vitamin E-free diet of controls.

4. Inhibitors

A great variety of substances has been studied regarding the inhibition of ChE (see AMMON, 1943; also ZIPF, 1942). A list of such substances, not previously published, is found in Table 4. The more important characteristics of the various "anticholinesterases"¹ will now be discussed briefly.

a) *Urethanes.* Of all the agents which inhibit ChE activity none (except DFP and HTP, p. 34) can compare in potency or specificity with *physostigmine* (eserine), the most important alkaloid derived

¹ The word "anticholinesterase" is frequently used, especially in pharmacological literature, for designating a drug which exerts its pharmacological activity by inhibiting ChE. The existence of a true anticholinesterase, that is, an immune body (antibody) produced by animals as the result of parenteral injections of ChE, has not yet been demonstrated.

from the calabar bean. In minute amounts (10^{-6} M) physostigmine protects ACh from enzymic hydrolysis and its pharmacological action is probably accounted for by this. The enzyme is not destroyed by physostigmine, but only combines reversibly with the drug. *In vitro*, physostigmine may be freed from this combination by dialysis. *In vivo*, it is released from this combination by breakdown of the drug or its excretion in the urine.

STRAUS and GOLDSTEIN (1943) and GOLDSTEIN (1944) have studied, in more detail than previous investigators (*e.g.*, STEDMAN & STEDMAN, 1931; EASSON & STEDMAN, 1936; ZELLER, 1942 b; EADIE, 1942), the mechanism of enzyme-inhibitor-substrate reactions, illustrated by the ChE-physostigmine-ACh system. The inhibition of ChE was said to be competitive and one molecule of physostigmine (or ACh) combines with one centre of ChE (horse serum) (see Chapter IX). The dissociation constant of the enzyme-inhibitor complex was found to be 3.11×10^{-8} . — The quantitative relation between the dosage of physostigmine and the inhibition of ChE activity in the blood serum (dog) was studied by KRAEYER, GOLDSTEIN and PLACHTE (1944).

It has been suggested (STEDMAN & STEDMAN, 1931) that the anti-ChE action of physostigmine is due to the urethane group. Also other urethanes (*e.g.*, prostigmine and miotine) have a similar effect compared with that of physostigmine; urethane itself, however, is very little active. But BLOCH (1939) found that phenol bases containing no urethane group have actions like physostigmine. And, according to MASSART and DUFAT (1940 c), the inhibiting action is due to a dissociating and substituted ammonium group (*cf.* below).

ELLIS *et al.* (1943) studied the pharmacological and chemical properties of physostigmine together with its breakdown products. As a method for the estimation of physostigmine at low concentrations (10^{-8} to 10^{-6} M) these authors proposed the inhibition of horse-serum ChE.

In addition to acting through its anti-ChE property, physostigmine is also said to exert a direct action on muscles. Moreover it is known that the inhibition of ChE does not itself completely explain the effect of physostigmine (*cf.* MANNING, LANG & HALL, 1937; KAHANE & LÉVY, 1937 a; MENG, 1940; CANTONI & LOEWI, 1944; BABSKY & KORENEVSKAJA, 1946).

The pharmacological actions of *prostigmine*, a synthetic alkaloid, are qualitatively similar to those of physostigmine. This is also true for its probable mechanism of action, *i.e.*, the inhibiting effect on ChE.

Prostigmine is employed in myasthenia gravis with beneficial results and gives better clinical results than physostigmine. However, it is not clear whether these results can be ascribed to the anti-ChE property of prostigmine. The serum ChE is markedly decreased by therapeutic concentrations of prostigmine, but whether this is also the case with muscle ChE is far from being established (*cf.* JONES & STADIE, 1939). More recent investigations on the pharmacology of prostigmine, however, suggest that several of the effects of this substance are not related to its anti-ChE action (MENDEZ & RAVIN, 1941; HEYMANS, 1946; RIKER & WESCOE, 1946).

Analogues of prostigmine have been prepared by AESCHLIMANN and STEMPEL (1946) and their activities determined. With these substances LEHMANN (1946) obtained a quantitative relationship between the inhibiting effect and "curare-like" potency, also shown by BÜLBRING and CHOU (1947). The anti-ChE activities of such substances have also been studied by BLOCH (1939), and HAWKINS and GUNTER (1946).

b) *Quaternary and tertiary ammonium bases*. The differences in anti-ChE action of the quaternary and tertiary amines have been investigated by SANZ (1945). It was observed that bases with quaternary

TABLE 4. *Substances Studied for Influencing Cholinesterase Activity*
 Serum ChE has been considered in the first place. +: inhibition; -: no action; a: activation.

| Substances | Action | References | Substances | Action | References |
|--|--------|---|---|--------|---|
| <i>Urethanes</i> | | | <i>Amines and amides</i> | | |
| Physostigmine (eserine) | ++ | See p. 28 | Cadaverine | + | 677 |
| Prostigmine (neostigmine) | +++ | See p. 29 | Urea | + | 206, 18; cf. 258, 205 |
| Miotine | ++ | 382, 564, 154 | Barbiturates | ? | 62, 206, 258, 5, 568, 569, 259 |
| Urethane | (+) | 206, 205, 18; cf. 258 | | | |
| <i>Quaternary ammonium bases</i> | | | Guanidine-HCl | - | 418, 416, 605, 340 |
| Choline and derivatives | + | 527, 689, 205, 18, 157, 142, 143; cf. 599 | Agmatine | (+) | 677 |
| Muscarine | ++ | 14, 15, 309 | Amino acids: glycine, alanine, etc. | ? | 35 |
| Betaine | ++ | 62 | Histamine | (+) | 15, 58, 656, 658, 18, 265, 190, 474, 257 |
| Methylene blue | ++ | 205, 379, 380, 508, 516, 119, 313 | | | |
| Oxazine derivatives | + | 516 | Thyroxine, 3,5-diiodotyrosine. | - | 304 |
| Phenazine derivatives | ++ | 205, 508, 516, 313 | Tryptophan | - | 35 |
| Acridine derivatives | ++ | 508, 119 | p-Aminobenzoic acid | (+) | 685, 677, 678, 351 |
| Curare | ? | 248, 249, 278, 299, 205, 511, 389 | Sulphanilic acid | - | 512 |
| | | 248, 529 | Sulphonamides: sulphanilamide, sulphathiazole, irgamide, etc. | + | 685, 677, 678; cf. 119, 517, 512 |
| β -Erythroidine | - | 527 | Atoxyl (arsanilic acid) | + | 14, 15, 618, 631, 632; cf. 496, 205 |
| Tetramethyl ammonium chloride | + | 564, 158 | | | |
| <i>Tertiary amines</i> | | | Adrenaline; sympathol, ephedrine, benzedrine, pervitine, etc. | ? | 14, 305, 309, 489, 304, 649, 205, 498, 512, 691, 535, 18, 294, 296, 59, 487 |
| Eseroline | ? | 205, 59 | | | |
| Geneserine | ++ | 583 | Phenanthrene amino alcohols | ++ | 668 |
| Bufotenine | ++ | 648 | Chloroalkyl amines | ++ | 603 |
| Atabrine | ++ | 72 | Arsines: lewisite, etc. | + | 57, 603, 55, 235 |
| Paludrine | (+) | 309, 564, 59 | Azo compounds: methyl orange, etc. | - | 508, 516 |
| Hordenine | + | | Chrysoidine | + | 379, 380 |
| Novocaine (procaine); larocaine, tutocaine, pantocaine, panthesine, nupercaine; phenacaine (holocaine) | ++ | 205, 517, 17, 23, 5, 18, 142, 143, 255, 351, 474, 188, 193, 474 | Diazonium compounds | + | 516 |
| | | 347, 309, 304, 23, 574, 18, 474, 59, 71, 193 | <i>Nitrogen-heterocyclic compounds</i> | | |
| Cocaine; psicaine, ecgonine | + | | Urotropine | (+) | 205, 305 |

| | | | | | |
|---|-----|--|---|-----|---|
| Atropine; hyoscyamine, tropine | ? | 14, 15, 527, 304, 305, 309, 139, 672, 59, 131 | Spartine | ++ | 305, 59; cf. 205 |
| Scopolamine | — | 59, 191 | Strychnine | + | 14, 15, 437, 440, 574, 305, 59; cf. 205, 483 |
| Nicotinamide | (+) | 14, 691, 558 | Veratrine | ? | 181, 59, 462, 317 |
| Coramine | — | 691 | Yohimbine | ++ | 59 |
| Nicotine | + | 440, 462; cf. 205, 59, 511 | Ibogaïne | ++ | 645, 59, 626 |
| Arecoline | — | 59 | Buxine (from <i>Buxus sempervirens</i>) | + | 639 |
| Cytisine | (+) | 59 | <i>Alkyl fluorophosphates</i> | | |
| Pelletierine | ++ | 59 | Di-isopropyl fluorophosphate (DFP) | ++ | 4, 387, 383, 126, 461, 109, 238, 526, 86, 78, 268, 239, 275, 254, 453 |
| Conine | ++ | 59 | | | |
| Indole | + | 649; cf. 119 | <i>Alkyl polyphosphates</i> | | |
| Morphine; codeine, thebaine, etc. | ++ | 292, 297, 205, 319, 62, 580, 145, 685, 152, 59, 267; cf. 163, 304, 264, 411, 191, 142, 143 | Hexaethyl tetraphosphate (HTP) and tetracthyl pyrophosphate | ++ | 150, 109 |
| Colchicine | ++ | 59; cf. 205 | <i>Alcohols, phenols, aldehyds, acids, etc.</i> | | |
| Apomorphine | ++ | 62, 319, 59 | Methanol, ethanol | (+) | 62, 414, 206, 166; cf. 10, 258 |
| Papaverine, narcotine, narceine | ++ | 319, 59, 142, 143, 411 | Avortin | ? | 7, 10, 5 |
| Emetine | ++ | 319, 59 | Propanol, isobutyl alcohol, etc. | (+) | 414 |
| β -Naphthylquinoline iodomethylate | ++ | 563 | Aryl alcohol | (+) | 206; cf. 258 |
| Quinine | ++ | 496, 14, 15, 618, 205, 278, 440, 648, 512, 59, 462; cf. 269, 498 | Propanediol | — | 161 |
| Cinchonidine and cinchonine | ++ | 59 | Alival | — | 304 |
| Ergot alkaloids: ergotamine, etc. | ++ | 347, 14, 15, 95, 205, 524, 463, 59 | Diethyl ether | + | 7, 610, 414, 166, 267, 422 |
| Antipyrine and other pyrazolone derivatives | + | 685, 678, 142, 143 | Ethyl nitrate | — | 304 |
| Imidazole derivatives: priscol, ofrivine, privine | + | 561 | Chloroform | ++ | 574, 62, 610, 414; cf. 259, 422 |
| Histidine | a | 35, 187 | Mustard gas | (+) | 603 |
| Antergan, antistine, etc. | + | 265, 474 | Formaldehyde | + | 121, 662, 205 |
| Pilocarpine | ? | 163, 14, 15, 205, 18, 304, 309 | Paraldehyde | + | 62 |
| Thiouracil | — | 159 | Chloral hydrate | + | 206, 258, 62, 10 |
| Aneurine | + | See p. 15 | Chloralose | + | 108; cf. 7 |
| Caffeine, theobromine | + | 292, 304, 62, 685, 462, 142, 143 | Glucose, fructose, saccharose | — | 304; see p. 28 |
| Pteroylglutamic acid (folic acid) | a | 140 | Starch, gum arabic | (+) | 495 |
| Metrazol (cardiazol) | — | 205, 575, 248, 691; cf. 524 | Malic acid (L- and DL-) | — | 301 |
| | | | Fumaric acid | + | 516 |
| | | | Phenol | + | 574 |
| | | | 2,4-Dinitro phenol | — | 62; cf. 511 |

TABLE 4 (cont.)

| Substances | Action | References | Substances | Action | References |
|-----------------------------------|--------|-----------------------------|--|--------|----------------------------|
| Cresol | + | 292 | Pituitary hormones: | | |
| Tri-o-cresyl phosphate | ++ | 74, 76, 272, 401; cf. p. 34 | Gonadotropic hormone, vaso- | | |
| Benzyl and salicyl alcohol | (+) | 18 | pressin | (+) | 611 |
| Salicylic acid, Na-salt | - | 62 | Hypophysis, pituglandol.... | + | 489, 321 |
| DDT | + | 646 | <i>Antibiotics</i> | | |
| Cyclopropane | - | 5 | Penicillin | (+) | 194 |
| Hexetone | ++ | 691 | <i>Anions</i> | | |
| Cholesterol | (+) | 690 | F ⁻ | ++ | 382, 496, 321, 278, 298, |
| Bile acids, Na-salts | + | 583, 69; cf. 556 | | | 205, 440, 18, 375, 376, |
| Strophanthin, K- | + | 691, 130, 139, 137, 384 | | | 151, 511 |
| ^a G- (ouabain) | + | 423, 628 | Br ⁻ , J ⁻ | (+) | 189 |
| Digitoxin, lanatosid C, scillaren | - | 423, 628 | AsO ₃ ⁻ , P ₂ O ₇ ⁴⁻ , C ₂ O ₄ ²⁻ , citrate, | | |
| tartrate | | | | ++ | 375, 376, 298, 18, 189; |
| <i>Thiol reagents</i> | | | CN ⁻ | - | cf. 205, 157, 511 |
| Copper, iodoacetic acid, alloxan, | + | 455, 456; cf. 57, 235 | SCN ⁻ | - | 174, 292, 18 |
| etc. | ? | 516, 654, 35 | <i>Inorganic salts</i> | | 375, 376 |
| Cystine | + | 654 | Li | + | 189 |
| Cysteine | + | 584 | Na | - | 527, 304, 151; cf. 10, 189 |
| Oxygen, o-iodobenzoate | - | 56; cf. 651 | K | ? | 527, 205, 189, 157; cf. |
| BAL (2,3-dimercaptopropanol). | (+) | | | | 402; see Chapter IX |
| <i>Vitamins</i> | | | NH ₄ Cl | + | 527 |
| Vitamin B ₁ | + | See p. 15 | Ca, Mg | a | See p. 27 |
| Vitamin C | ? | 516, 18, 533, 237, 195, | Ba, Zn, Hg, B, Al, P, As, Sb, Bi, | | 151, 443, 189, 192, 195, |
| | | 189; cf. 304 | Ag, Au, Cu, Pb, Ni, Co, Fe .. | | 264, 631, 632, 455, |
| Vitamin E | a | 75 | | | 456, 376 |
| Vitamin K | + | 613 | <i>Other substances</i> | | |
| <i>Hormones</i> | | | Deuterium oxide | + | 83 |
| Thyroxine | ? | 18; cf. 30, 658, 159 | Radium emanation | ? | 304, 310 |
| Parathormone | - | 492 | Proteose peptone | + | 318; cf. 179 |
| Insulin | ? | 533, 505, 504; cf. 84 | Insecticides, various sorts of.. | - | 511 |
| Vagotonine | ++ | 84, 541, 487 | Tetanus toxin | + | 206, 18, 658, 642, 499 |
| Kalikrein (pure preparation).. | - | 658 | Diphtheria toxin | + | 18, 642 |
| Padutin | + | 321 | Tuberculin | (+) | 198 |
| Cortin | ? | 205 | Vegetable oils | + | 103 |
| Sex hormones: estrone, etc.... | (+) | 611, 412; cf. 161; see p. 8 | | | |

ammonium groupings are very strong inhibitors of the specific ChE, whilst the tertiary amines inhibit the non-specific esterase more powerfully.

All *quaternary ammonium bases* are strong inhibitors of ChE (except betaine), thus choline (see Chapter X) and its derivatives inhibit the enzyme. The action of neurine, not previously studied in this connection, is considered in Chapter XI. Methylene blue and other basic dyes containing a dissociable quaternary ammonium ion, are potent inhibitors of ChE. The leuco forms, which are not quaternary ammonium bases, have no inhibiting effect. The anti-ChE action of methylene blue is discussed more fully in Chapter XI.

In addition to the salts of physostigmine and miotine other *tertiary amines* have been investigated for their anti-ChE action. Bufotenine, the structure of which is similar to that of physostigmine, is a very active inhibitor. Atebrine inhibits ChE strongly; on the other hand, the new antimalarial drug paludrine, has little affinity for ChE. Cocaine, novocaine, and other local anaesthetics belonging to the same category potentiate ACh action strongly. No connection between chemical constitution and anti-ChE action appears to exist in this case, but a certain correlation is to be found between anti-ChE activity and pharmacological action.

c) *Amines and amides*. Prolonged administration of barbiturates causes serum ChE to reach low values. This action is said to be due to a lowering of the enzyme concentration rather than to a competitive or non-competitive inhibition. In addition the ChE activity of the spinal cord and muscle are considerably reduced, although the activity of brain tissue remains unchanged.

The action of *p*-aminobenzoic acid and the sulphonamides on ChE has been investigated, especially by ZELLER, who found definite inhibition in all cases. The affinity of *p*-aminobenzoic acid for ChE was considerably greater than that of the sulphonamides. The degree of inhibition was different for various ChE preparations. Other investigators did not find any action with the sulphonamides.

Inconsistent results have also been reported with adrenaline and its related substances. Adrenaline increases the response of the muscle to ACh (TORDA & WOLFF, 1946), but this increase is probably due to a different mechanism than that operating during sensitisation of muscle to ACh by physostigmine. In the nervous system adrenaline, in low concentrations, augments the effect of ACh, whilst in higher concentrations it depresses it (review: BURN, 1945). These effects may be explained by an anti-ChE action of adrenaline or its breakdown products, but such an explanation does not cover all the observed facts in this complex problem.

WRIGHT (1946) found that the phenanthrene amino alcohols are very effective inhibitors of the ChE of human blood plasma (non-specific ChE), the dipropylamino derivatives having the highest activity. Much higher concentrations are required to inhibit the erythrocyte enzyme (specific ChE).

The action of chemical vesicants on ChE (brain, skin, serum) was investigated by THOMPSON (1947) (cf. also the review by PETERS, 1947). The enzyme was strongly inhibited by certain chloroalkyl amines, less by mustard gas and various arsines, still less by lewisite. THOMPSON thinks that some of the pathological effects produced by these vesicants may be due, in part, to an inhibition of the ChE at cholinergic nerve-endings. The British Anti-Lewisite (BAL) seems to have a weak inhibiting effect on ChE (BARRON, MILLER & MEYER, 1947).

d) *Nitrogen-heterocyclic compounds*. A great variety of alkaloids has been investigated with regard to their anti-ChE activities. A comprehensive study is presented by BEAUJARD (1944), a synopsis of which is given by VINOENT and BEAUJARD (1943).

Atropine and its isomer hyoscyamine, inhibit ChE only slightly, although most probably this effect plays no significant rôle in the action of ACh.

Morphine and its related substances have relatively strong anti-ChE actions, the inhibition being found for both specific and non-specific ChE. Quinine, on

the other hand, has less affinity for specific ChE than for other esterases (Chapter XI).

Antipyrine and other pyrazolone derivatives are inhibitors of ChE. According to ZELLER, the non-specific ChE is more strongly inhibited than the specific one. On the other hand, caffeine is said only to inhibit the specific ChE (Chapter XI).

A very strong inhibitor of ChE is the alkaloid from *Tabernanthe iboga*, ibogaine, the action of which is said to be of the same order as that of physostigmine.

e) *Alkyl fluorophosphates*. These recently discovered substances act as powerful inhibitors of ChE both *in vivo* and *in vitro*. The dimethyl ester was the first (1941) to be investigated (ADRIAN, FELDBERG & KILBY, 1947). The most active one is di-isopropyl fluorophosphate (DFP) which produces an inhibition even in a concentration of 10^{-11} M (DIXON *et al.*; for references, see McCOMBIE & SAUNDERS, 1946) and is the most powerful and specific enzyme inhibitor known. In contrast to the inhibition of ChE by physostigmine, the action of DFP is progressive and irreversible (MAZUR & BODANSKY, 1946). The inhibition is said to be specific for ChE and selective for the non-specific esterase (HAWKINS & MENDEL, 1947; cf. BODANSKY, 1946). These results have been confirmed in various quarters and the investigation of these interesting substances is still progressing.

Extensive pharmacological and biochemical studies have been carried out on DFP during the War, especially in America (Edgewood Arsenal). The reader is referred to the vols. 87—91 (1946—1947) of the *J. Pharmacol.* (papers by COMROE, GILMAN KOELLE, and others) and to the papers by GROB, LILIENTHAL, HARVEY and JONES (1947).

f) *Alkyl polyphosphates*. Another recently discovered group of potent inhibitors of ChE are the tetraethyl pyrophosphate and hexaethyl tetraphosphate (HTP), introduced as insecticides during the War by the Germans. The anti-ChE activity of these compounds was shown by DuBOIS and MANGUN (1947) and CHADWICK and HILL (1947). They are said to be more active than DFP and their effects may be at least in part due to their inhibition of ChE.

g) *Alcohols, phenols, aldehyds, acids, etc.* Methanol and ethanol in high concentrations inhibit ChE. Chloroform is 40 times more active than ether and 1/500 as active as physostigmine.

Among the three possible isomers of tricresyl phosphate, the *o*- and *m*-derivatives inhibit the serum ChE *in vitro*, the *p*-derivate does not. *In vivo*, only tri-*o*-cresyl phosphate acts as an anti-ChE.

The sodium salts of a series of bile acids are inhibitors of ChE which probably explains the observation that bile is not ChE active. The characteristic lowering effect on the blood pressure caused by bile salts, however, is not due to an inhibition of ChE (SCHACHTER & DWORKIN, 1942).

h) *Thiol reagents*. As shown by NACHMANSOHN and LEDERER (1939 a, b) ChE belongs to those enzymes the activity of which is dependent upon free sulphhydryl groups. Thus it was found that substances capable of transferring SH groups to SS groups have anti-ChE properties. The influence of cystine on ChE is discussed in Chapter XI.

In a study on the effect of high oxygen pressures upon various enzymes, STADIE, RIGGS and HAUGAARD (1945) have shown that ChE is an example of an SH enzyme resistant to oxygen at increased pressure.

i) *Vitamins*. The actions of vitamin B₁ (aneurine) (p. 15) and vitamin E (p. 28) respectively on ChE have been reviewed above. Inconsistent results have been reported for the action of vitamin C on ChE activity (cf. p. 28). Menadione (a vitamin K analogue) in concentrations more than 10^{-3} M seems to inhibit the esterase.

j) *Hormones*. The only hormone which has a significant effect on serum ChE is vagotonine, a preparation from pancreas which increases vagal tone and slows

the heart. Vagotonine is said to combine with the same group of the enzyme molecule as does physostigmine (POLONOVSKI, SANTENOISE & PELOU, 1943).

k) *Anions*. ChE is greatly inhibited by fluoride, oxalate, citrate, arsenite, and pyrophosphate. Presumably these effects are due to a removal of Ca^{2+} or Mg^{2+} which are said to be activators of ChE. Cyanide has no effect on serum ChE.

l) *Metal salts*. FROMMEL *et al.* (1943, 1944, 1946) have made an especial investigation into the effect of various inorganic salts on ChE both *in vitro* and *in vivo*. None of the substances investigated, summarised in Table 4, has thrown any new light on the nature of ChE (cf. p. 27).

m) *Other substances*. While KARK (1938) did not find any action of RaEm on serum ChE, KEESER (1943) has reported that the activity of blood is decreased after drinking water containing RaEm. Both tetanus toxin and diphtheria toxin are said to inhibit ChE activity, but according to AMMON (1943) the toxins themselves have no effect, the inhibition being due to impurities.

PART II

CHAPTER III

METHODS

A. MANOMETRIC METHOD IN DETERMINING CHOLINESTERASE ACTIVITY

1. Use of Warburg Manometric Apparatus

The ChE activity was measured by the Warburg manometric method, in the modification used here, described by the author (Augustinsson, 1944) (cf. p. 23). The method is based on the manometric estimation of the volume of CO_2 evolved from a bicarbonate-containing system by the acid formed in the ester hydrolysis.

Conical flasks, each of 15–20 ml. volume, with one side bulb were employed. The flask constants were determined by the calibration method using mercury. The manometers were filled with Brodie's solution, containing 23 g. NaCl and 5 g. sodium choleate in 500 ml. water; a few drops of an alcoholic solution of thymol were added. The fluid was coloured with methyl violet; indigo carmine has been used also, but it tends to decompose in the manometer. The density of the solution is 1.034, and 10 000 mm. Brodie corresponds approximately to 760 mm. Hg.

The flasks were carefully cleaned; grease was removed with ether. They were washed with water and placed in cleaning solution overnight. The cleaning solution was prepared by dissolving 50 g. potassium dichromate in 35 ml. hot water and adding conc. H_2SO_4 to 1 litre. Finally, the flasks were rinsed several times with distilled water and dried in drying chamber.

The grease used in lubricating the joints was anhydrous lanoline.

The flasks and manometers were shaken at about 90 complete oscillations per minute. The shaking amplitude was about 7 cm. In most cases, the temperature of the water thermostat was $37.5 \pm 0.05^\circ\text{C}$ unless otherwise stated. For convenience, the temperature of the thermostat at the Biological Station of Roscoff was maintained at $25.0 \pm 0.1^\circ\text{C}$.

2. Measurement of Activity

The volume of the reaction mixture has always been 2.00 ml. In the main compartment of the flask 1.60 ml. of the substrate solution was placed and in the side bulb 0.40 ml. of the enzyme

solution or a mixture of 0.20 ml. of the enzyme solution and 0.20 ml. of the inhibitor solution. Substrate and enzyme preparations were dissolved in a bicarbonate-buffer solution (see p. 38).

The hydrolysis was carried out in a gas mixture of 95 per cent N_2 and 5 per cent CO_2 by volume. $CaCO_3$ is formed if the solution is not in equilibrium with at least 5 per cent CO_2 , when the optimum conditions are changed and the evolution of CO_2 is disturbed. The solutions were saturated with the gas mixture and the flasks filled after they had been attached to the manometers. Before the enzyme solutions were mixed with the contents of the main portion of the flask the temperature equilibrium was attained by shaking in the water thermostat for about 15 minutes.

The shaker was stopped. The first manometer was read, lifted from its mount, the contents were mixed at zero time, and the manometer was placed back on its mount and the shaker started again. At one minute intervals the contents of the other flasks were similarly mixed. Usually, each series included six to eight experiments. Each manometer was read at 6 to 10 minute intervals, one minute between each manometer-reading. Readings were made continuously for 40 to 60 minutes.

In all experiments a thermobarometer was used, filled with 2.00 ml. water or with the same volume of the substrate solution of the same concentration as that in the reaction mixtures. Corrections for thermobarometer changes (due to alterations in temperature and pressure, and eventually to the non-enzymic hydrolysis of the substrate) were made.

The results were recorded in tabular form, the shape of which is shown in the Appendix (p. 182). The amount of CO_2 expressed in μ l. was plotted against time. The initial slope of the curve (in most cases a straight line), minus the slope of the curve for non-enzymic hydrolysis was then taken as an expression of the enzyme activity (see p. 53). The extrapolated 30 min. value ($= a_{30}$), minus the amount of CO_2 evolved during the same time period by non-enzymic hydrolysis, has been used as unit in expressing the esterase activity and symbolised by b_{30} . Expressed in μ mol. substrate hydrolysed during the same time period, the activity is $b_{30}/22.4$ μ mol. One μ l. CO_2 corresponds to 8.1 μ g. ACh chloride, or 1.0 mg. ACh chloride = 123.5 μ l. CO_2 .

The reliability of the method has been determined in some experiments. In a series of 18 determinations of the activity of a ChE preparation from *Helix* blood, the standard deviation was 2.0 μ l. and the mean 131.2 ± 0.5 μ l. CO_2 .

B. DETERMINATION OF pH

The glass electrode has been used for pH measurements; the accuracy of the values was ± 0.01 pH unit. In some earlier experiments a less convenient hydrogen electrode was used, the accuracy of which was somewhat lower. Indicator paper has been employed also in

some preliminary experiments when greater exactness was not necessary. This has been the case in the determination of enzyme stability at various pH values and in some experiments of purifying ChE.

CHAPTER IV

MATERIALS

A. BUFFER SOLUTIONS

In most cases the experiments have been carried out in a bicarbonate-RINGER's solution (R_{30}), the composition of which is given in Table 5. This solution, made by substances of highest purity and distilled water, was used for dissolving the substrate and diluting the enzyme preparations. In some cases it was also employed in extracting the enzyme from the disintegrated tissue. Fresh R_{30} was always prepared before use, since the solution deteriorates if kept. The pH of R_{30} , saturated with the N_2 - CO_2 gas mixture, was 7.40.

In experiments of determining the effects of K^+ and Ca^{2+} or of other substances on ChE, the substrates were dissolved in a solution containing only $NaHCO_3$, of the same concentration as that of the R_{30} .

The phosphate buffer, used in some preparatory experiments, was made from Na_2HPO_4 and KH_2PO_4 and had pH 6.60.

TABLE 5. *Composition of the Bicarbonate RINGER's Solution (R_{30})*

| Solution | %
(w/v) | ml. | R_{30}
Molarity |
|----------------------------|------------|-----|-----------------------|
| NaCl | 0.90 | 100 | 1.15×10^{-1} |
| $NaHCO_3$ | 1.26 | 30 | 3.36×10^{-2} |
| KCl | 1.20 | 2 | 2.40×10^{-3} |
| $CaCl_2 \cdot 6H_2O$ | 1.76 | 2 | 1.20×10^{-3} |

B. SUBSTRATES

The substrates used are listed in Table 6 which shows the abbreviations used in this paper, molecular weights and stabilities of the various substances.

For each experiment fresh solutions of the substrates (in R_{30}) were made and the spontaneous hydrolysis determined. A 0.25 per cent (w/v) solution (No. 3) was employed unless otherwise stated. When the activity-substrate concentration relationships were studied, six solutions of each substrate were used with the following concentrations:

| Solution No. | 1 | 2 | 3 | 4 | 5 | 6 |
|--------------------------------|------|------|------|-------|-------|--------|
| Percentage concentration (w/v) | 2.50 | 0.75 | 0.25 | 0.075 | 0.025 | 0.0075 |

The final molar concentrations of the substrates after mixing the enzyme solutions or R_{30} (in the studies of non-enzymic hydrolysis) with the substrate solutions are found in Table 9 (p. 48—49).

Acetylcholine was usually used in the form of the chloride, but the bromide and iodide have been employed in some experiments.

ACh bromide and iodide are more convenient for laboratory experiments than the chloride, as they are much less hygroscopic than the latter. The two salts, however, have been available only in small quantities and the ACh perchlorate, which is said to be completely non-hygroscopic (CARR & BELL, 1947), has not been available at all. In most cases the bromide is enzymically hydrolysed at the same or at a somewhat lower rate than the chloride. The iodide has always been found to decompose at a lower rate (75—85 per cent of the hydrolysis of the ACh chloride). In such comparative experiments, both the specific (erythrocytes, brain) and non-specific ChE (serum) and other types of this esterase (*Helix* blood, the dart sac) have been used. In experiments performed with horse serum, GLICK (1938 c) obtained no effect upon the enzymic hydrolysis of ACh when the associated anion in the substrate was changed.

As regards acetylsalicylic acid as substrate, the acid was neutralised with 0.1-M NaOH and used immediately after neutralisation. The tributyrin solutions (emulsions) were thoroughly shaken for one hour before use.

C. OTHER CHEMICALS USED

1. Substances Used in Preparatory Experiments

The following chemicals were used in preparatory experiments:

Distilled water has been used as solvent in all experiments.

Ammonium sulphate of high purity was employed in the purification of ChE from various sources.

Hydrochloric acid, 0.1 M.

Collodion, 4 per cent, according to the *Swedish Pharmacopoeia* was used in preparing membranes for dialysis.

Heparin (VITRUM), 1 per cent solution, was employed for delaying the clotting of blood; 1 ml. in 100 ml. blood.

Sand. Fine sea sand was washed with concentrated H_2SO_4 , then several times with distilled water, and dried in drying chamber.

Infusorial earth (COLEMAN & BELL) was used in some preliminary experiments in order to adsorb the enzyme in purification procedures.

Lysolecithin, prepared from egg-yolk,¹ was employed in a 0.1 per cent solution of 1.26 per cent bicarbonate solution.

2. Substances Used for Influencing Cholinesterase Activity

The substances of highest purity were dissolved in distilled water. The concentrations of the solutions were as shown in Table 7. In using these solutions, 0.20 ml. was placed in the side bulb together with 0.20 ml. of the enzyme preparation. The solutions of physostigmine salicylate were always made immediately before use in each case.

¹ This preparation was kindly placed at my disposal by the Department of Cell Physiology at Wenner-Gren's Institute for Experimental Biology, University of Stockholm.

TABLE 6.

| Substrate | Abbreviation | Formula |
|--|-----------------|---|
| Acetylcholine chloride.... | ACh | $[(CH_3)_3N-CH_2-CH_2-O-COCH_3]Cl$ |
| Acetylcholine bromide.... | | $[(CH_3)_3N-CH_2-CH_2-O-COCH_3]Br$ |
| Acetylcholine iodide..... | | $[(CH_3)_3N-CH_2-CH_2-O-COCH_3]I$ |
| DL-Acetyl- β -methylcholine chloride (Meeholyl)..... | MeCh | $[(CH_3)_3N-CH_2-CH(CH_3)-O-COCH_3]Cl$ |
| Carbaminoylcholine chloride (Doryl, Lentin).... | CbCh | $[(CH_3)_3N-CH_2-CH_2-O-CONH_2]Cl$ |
| Benzoylcholine chloride... | BzCh | $[(CH_3)_3N-CH_2-CH_2-O-COC_6H_5]Cl$ |
| N-Acetyl- <i>p</i> -aminobenzoylcholine chloride..... | AAmBzCh | $[(CH_3)_3N-CH_2-CH_2-O-COC_6H_4NH-COCH_3]Cl$ |
| Salicylcholine chloride | SaCh | $[(CH_3)_3N-CH_2-CH_2-O-COC_6H_4OH]Cl \cdot H_2O$ |
| Acetylsalicylcholine chloride | ASaCh | $[(CH_3)_3N-CH_2-CH_2-O-COC_6H_4O-COCH_3]Cl$ |
| Acetylneurine chloride hydrochloride..... | AA _n | $[C_{14}H_{19}O_2N_4S]Cl \cdot HCl$ (cf. Table 2) |
| Acetylsalicylic acid | ASa | $HOOC-C_6H_4-O-COCH_3$ |
| Tributyrin | TB | $C_3H_5O_2(CO-CH_2-CH_2-CH_3)_3$ |
| Ethyl acetate | EA | $C_2H_5O-COCH_3$ |

TABLE 7. *Substances Used for Influencing Cholinesterase Activity*

| Substances | Mol.wt. | Concentration | |
|--------------------------------|---------|--------------------|----------------------------|
| | | Solution used
% | Reaction mixture
M or % |
| Physostigmine salicylate | 413.46 | 0.0015 | 3.63×10^{-6} M |
| Potassium chloride | 74.55 | 7.45 | 0.10 M |
| Lithium chloride | 42.40 | 4.24 | 0.10 M |
| Clupeine | — | 0.50 | 0.05 % |
| Gum arabic | — | 0.50 | 0.05 % |
| Methylene blue | 373.90 | 0.15 | 4.01×10^{-4} M |
| Caffeine | 212.21 | 0.15 | 7.07×10^{-4} M |
| Quinine hydrochloride..... | 396.91 | 0.15 | 3.78×10^{-4} M |
| DL-Cystine | 240.29 | 0.15 | 6.24×10^{-4} M |
| Neurine bromide..... | 166.07 | 0.15 | 9.03×10^{-4} M |
| Choline chloride | 139.63 | See Chapter X | |

D. ENZYME PREPARATIONS

Enzyme preparations have been made from tissues and body fluids of various animals listed in Table 8. The material has been placed at my disposal by the Departments of Pathology, Physiology,

Substrates

| Mol. wt. | Stability | | |
|----------|------------------------------------|----------------------|--|
| | in air | in water | |
| 181.66 | Very hygroscopic | Very unstable | HOFFMANN-LA ROCHE & Co. |
| 226.12 | Less hygroscopic than the chloride | Unstable | LIGHT & Co. |
| 273.13 | Non-hygroscopic | Unstable | LIGHT & Co. |
| 195.69 | Hygroscopic | Not very stable | MERCK & Co. |
| 182.65 | Non-hygroscopic | Fairly stable | BURROUGHS WELLCOME & Co. |
| 243.73 | Non-hygroscopic | Not very stable | HOFFMANN-LA ROCHE & Co. |
| 300.78 | Non-hygroscopic | Not very stable | Synthesised according to EULER, EULER and HASSELQUIST (1945) |
| 277.74 | Non-hygroscopic | Very unstable | Synthesised according to EULER <i>et al.</i> (1945) |
| 301.77 | Non-hygroscopic | Very unstable | LKB, Stockholm (cf. EULER, HASSELQUIST & HÖGGERG, 1944) |
| 379.31 | Non-hygroscopic | Not very stable | HOFFMANN-LA ROCHE & Co. |
| 180.15 | — | As Na-salt, unstable | BOFORS AB |
| 302.36 | — | Fairly stable | EASTMAN KODAK Co. |
| 88.10 | | Fairly stable | SCHERING-KAHLBAUM A. G. |

and Animal Breeding of the Royal Veterinary College, Stockholm, by the Marine Zoological Station of the Royal Academy of Sciences, Kristineberg, Sweden, and by the Biological Station, Roscoff, France. In most cases, fresh material has been used.

*1. Blood**a) Blood of Vertebrates*

Blood has been taken from man, horse, cow, guinea pig, fowl (cock), bony fish (*Labrus*), and dogfish shark (*Scyllium*). In some cases the blood was defibrinated by stirring immediately after withdrawal, centrifuged, and the serum and red blood cells used for further preparation. In other cases the blood was taken up in heparin (1 ml. in 100 ml. blood), centrifuged, and the plasma and cells used. It has been found that heparin has no influence on the ChE activity and that serum and plasma have the same activity. Accordingly serum and plasma have been used interchangeably without further comment.

As regards the method of collecting fish blood, the skin of the animals was removed on the ventral side at the site of the pectoral girdle. A hole was made in the girdle and the heart (*Labrus*) or sinus

TABLE 8. *Animals and Tissues from which Enzyme Preparations have been made*

| Animals | | Tissues | |
|---------------|---|--|--|
| Vertebrates | Man ♀ | Blood | Royal
Veterinary
College |
| | Horse | Blood | |
| | Cow | Blood, liver | |
| | Elephant | Brain, liver | |
| | Bear | Brain | |
| | Dog | Brain, muscle | |
| | Guinea pig | Blood, liver, kidney, intestine | Kristineberg |
| | Fowl (cock) | Blood, liver | |
| | <i>Gadus callarias</i> (cod) | Brain, muscle, liver, heart, tunica interna, gas-gland | Roscoff |
| | <i>Labrus berggylta</i> | Blood, brain, muscle, liver | Roscoff |
| | <i>Scyllium (Scylliorhinus) canicula</i> (dogfish shark) | Blood, brain, liver | |
| | <i>Squalus acanthias</i> (spiny dogfish shark) | Brain, muscle, liver, heart | Kristineberg |
| Invertebrates | <i>Raja radiata</i> (common ray) | Brain, muscle, liver, heart | Kristineberg |
| | <i>Myxine glutinosa</i> (hag-fish) | Brain, muscle, liver | Kristineberg |
| | <i>Balanoglossus clavigerus</i> | Proboscis, collar, "liver-sacs" | Roscoff |
| | <i>Paracentrotus lividus</i> | Eggs | Roscoff |
| | <i>Sepia officinalis</i> (cuttlefish) | "Liver" | Roscoff |
| | <i>Helix pomatia</i> (edible snail) | Blood, dart sac, etc. | Collected in the environs of Stockholm |
| | <i>Maia squinado</i> (spider crab) | Muscle | Roscoff |
| | <i>Spirographis Spallanzani</i> | Blood | Roscoff |
| | <i>Sagartia parasitica</i> | Parts with nerve net | Roscoff |
| | Marine animals belonging to various groups of invertebrates | Mostly whole animals | Kristineberg |
| | <i>Apis mellifica</i> (honey bee) | Venom | HOFFMANN-LA ROCHE |

venosus (*Scyllium*) cut. The blood was taken up in heparin and centrifuged. The experiments with fish blood were carried out at 25.0°C.

α) *Serum-ChE preparation*. The method in purifying serum ChE from horse has been described by the author (AUGUSTINSSON, 1944). The same method has been employed here with other vertebrate sera. The principle of the purification is as follows. At 50 per cent saturation of serum with ammonium sulphate practically all ChE remains in solution. The pH of this solution is lowered to about 4.5, when

the enzyme is separated from the greater part of the albumins which are precipitated. ChE is then precipitated by increasing the salt concentration to 70 per cent. The precipitate is dissolved in distilled water and the fluid dialysed in collodion membranes against distilled water. The preparations thus obtained had been purified 20—25 times and the yield was about 50 per cent.

β) *Erythrocyte-ChE preparation*. The blood cells were washed three times with 0.9 per cent NaCl solution and then haemolysed with distilled water to the same volume as the original blood volume. In the case of the shark blood the erythrocytes were washed with 2.0 per cent NaCl solution which acts as "physiologic salt solution" for this blood. For further preparation the haemolysates were diluted with the same volume of distilled water; the fish haemolysates were not diluted. From these solutions the erythrocyte membrane or stroma was flocculated by adjusting the reaction to pH 6.5—6.6 (or in some cases to about 5.5) with 0.1-M HCl (according to JONNES, 1932). The "ghosts" were washed twice with distilled water and twice with a slightly acid phosphate buffer (pH 6.60). These manipulations did not elute or alter the ChE activity of the precipitate. The fact was that the ChE of the blood cells was attached to the cell membrane. The results are found in Chapter VI (p. 57, Table 13, Fig. 2).

The "ghosts", washed with water and phosphate buffer, were shaken one hour with 2 volumes of a 0.1 per cent solution of lyssolecithin in a 1.26 per cent bicarbonate solution and 2 volumes of water. After centrifuging the whole enzyme activity was found in the centrifugate; about a half of the flocculation had gone into solution. The results of this procedure are found in Table 14 (p. 60).

b) *Blood of Invertebrates*

α) *Helix pomatia*. The edible snails were collected in the early autumn just before the winter sleep, and kept in a box with soil and leaves. In this captivity the animals shortly prepared for hibernation in the usual manner. No changes of the ChE activity in the blood and other tissues of the snails could be found in the first months.

A hole was made in the shell. The blue blood was drawn from the circulus venosus and kept in the refrigerator for months. Kept in this way, the ChE activity was changed only a little during a period of four months.

The ChE of *Helix* blood has been purified in the following manner. 30 g. ammonium sulphate was added to 100 ml. blood (55 per cent saturation). After about one hour's good stirring the precipitate was removed by centrifuging and the centrifugate discarded. The precipitate was dissolved in water, when a quite clear fluid was obtained. The fluid was dialysed against distilled water. The nitrogen contents of the different fractions have been determined by the KJELDAHL method and the protein constituents calculated. The results of these experiments are discussed in Chapter VI (p. 62).

For further purification the solution of the sulphate precipitate has been shaken with infusorial earth during one hour. The suspension was centrifuged and the ChE activity of the centrifugate determined. In order to elute the adsorbed enzyme the earth was suspended in 10 per cent ammonium-sulphate solution (p. 62).

β) *Spirographis Spallanzani*. This true tube-dwelling polychaete worm, belonging to the family Sabellidae, was caught in the summer and the tube removed. On the ventral side, at the junction between head and thorax regions, the skin was removed with care. The green blood, containing the respiratory pigment chlorocruorin in solution, was taken up with a fine pipette. One ml. blood was obtained from about 15 animals. The blood was diluted with the same volume of distilled water.

2. Tissues

Fresh material has been used generally. The tissues were carefully washed with water and minced. In a mortar 5 to 25 g. was taken for thorough grinding with washed sand. The disintegrated tissue was taken up with twice or four times as much bicarbonate-RINGER's solution. The mixture was shaken in a shaking machine for half an hour, centrifuged at a constant speed of 3 000 r. p. m., and the fluid decanted. Very different time intervals were needed to produce as clear a fluid as possible. The buffered suspensions were kept in the refrigerator and further preparations or analyses were made within two or three days. The activities of these tissue suspensions were generally not changed remarkably during two weeks, during which time all investigations were performed.

a) Tissues of Vertebrates

Brain. Dog brain has been used in most cases; brains have also been taken from elephant, bear, cod, *Labrus*, shark, ray, and *Myxine*. The brain was carefully washed with water to remove all blood. White and grey matter were prepared in some cases and the material treated according to the way described above.

Muscles have been used from dog, cod, *Labrus*, shark, ray, and *Myxine*.

Liver preparations were made from cow, elephant, guinea pig, cod, fowl, *Labrus*, shark, ray, and *Myxine*.

Kidney of ox and guinea pig was employed.

Intestine. Small intestines of guinea pig were washed with water and extracts prepared.

b) Tissues of Invertebrates

In some earlier investigations (at Kristineberg Zoological Station) with materials from invertebrates, the animals were frozen at -20°C in a freezing box immediately after they were caught. The frozen material was kept for some weeks and then melted and minced. Further preparation proceeded according to above. Most of

the animals collected in Roscoff were dissected immediately after the catch and preparations made in the usual manner for determinations there at 25.0°C.

Helix pomatia. After the blood was drawn from the animal (p. 43), the whole shell was removed and the body dissected. The following tissues have been studied: the head with the central nervous system, the foot, the digestive gland ("liver"), the liquid secreted by the "liver", the hermaphrodite gland (ovotestis), the mucous glands, and the dart sac without the dart.

Sepia officinalis. The bodies of the cuttlefish were dissected for preparing the "livers" (digestive glands).

Maia squinado. The spiny crabs were dissected for abdominal muscles.

Balanoglossus clavigerus. This burrowing Enteropneusta was caught on the beach at Morgat (France). The following material was taken: the proboscis, the collar-region, and the outgrowths of the alimentary canal (the "liver-sacs").

Sagartia parasitica. The upper end of the body of this anemone with the mouth, the circular muscles, and the circles of tentacles were prepared for extraction. These parts contain a well-developed diffuse nerve net.

Paracentrotus lividus. Centrifuged unfertilised and fertilised sea-urchin eggs at various stages were frozen and dried.¹ The dry powder was suspended in R₃₀, 1 g. in 8.0 ml. solution. The suspension was shaken for an hour and then centrifuged. The centrifugate was used in the determinations of ChE activity and nitrogen content by the KJELDAHL method.

Marine animals belonging to various groups of invertebrates. The investigations carried out with this material have been reported previously (AUGUSTINSSON, 1946 b).

c) Bee Venom

The venom of honey bees was in a dried and crude state and had not been sterilised by heating. It was dissolved in bicarbonate-RINGER's solution, in which the venom was found to be more soluble than in pure water. A 0.1 per cent solution has been employed in investigating the ChE activity of bee venom or, if the enzyme was not present, the influence of the venom on the ChE activity of other material.

¹ This material has been placed at my disposal by Dr. TRYGGVE GUSTAFSON, Wenner-Gren's Institute for Experimental Biology, University of Stockholm.

PART III

CHAPTER V

NON-ENZYMIC HYDROLYSIS

When biological methods (p. 23) are used in determining the ChE activity, it is not always necessary to consider the non-enzymic hydrolysis of the substrates. In these cases strong concentrations of the enzyme are used which consequently leads to a very high rate of the enzymic hydrolysis compared with that of the non-enzymic one. In the chemical method, as that used in the present investigation, the enzymic concentration is much lower. Because of that the enzymic hydrolysis is low and the non-enzymic one constitutes a considerable part of the total hydrolysis.

In every experiment therefore, it has been necessary to know definitely the rate of non-enzymic or spontaneous hydrolysis of the substrate solutions. A detailed study of this hydrolysis has been performed with all substrates. A rough idea of the stabilities of the various substrate solutions is given by Table 6. Table 9 specifies more accurately the stabilities and shows the non-enzymic hydrolysis expressed in $\mu\text{l. CO}_2$ per 30 minutes of all substrate solutions used in the experiments reported in the following. In Fig. 1 the hydrolysis at 37.5°C is plotted against the concentrations of the substrates, expressed in $-\log$ molar concentration ($= \text{pS}$). The concentrations are those finally attained after mixing the enzyme solutions or R_{30} with the substrate solutions.

In the determination of non-enzymic hydrolysis, the substrate solution in the main compartment of the flask was mixed with R_{30} of the side bulb.

A comparison of the hydrolysis in the solutions of acetylcholine chloride (1) (Fig. 1), bromide (2), and iodide (3) showed that the three salts give nearly the same hydrolysis-concentration curves. The hydrolysis increases rapidly in concentrations above 10^{-2} M. In a 0.1-M solution of ACh chloride, the half of the ester is hydro-

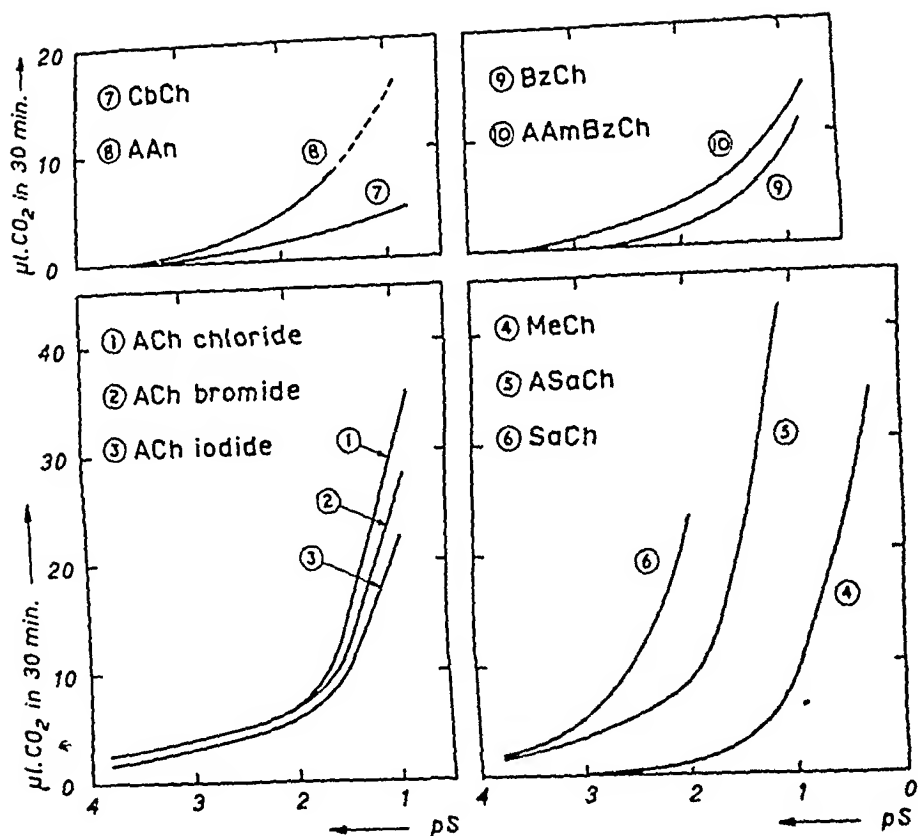


Fig. 1. Non-enzymic hydrolysis of substrate solutions as function of concentration at 37.5° C. pS = $-\log$ molar concentration. Data from Table 9.

lysed in 41 hours at 37.5°C. Also the bromide and iodide are hydrolysed spontaneously at a high rate; the half is hydrolysed in a 0.1-M solution in about 49 and 62 hours respectively at 37.5°C.

Much more stable are the solutions of benzoylcholine chloride (9) and its N-acetyl-*p*-amino derivate (10). Carbaminoylcholine chloride (7) is the most stable of the choline esters used in these experiments. A solution of the acetic-acid ester of aneurine (8) is somewhat less stable. Mecholyl (4) hydrolyses at about the same rate as does BzCh. The most unstable solutions are given by acetylsalicylcholine chloride (5) and salicylcholine chloride (6). A 0.1-M solution of the acetylsalicylic ester is destroyed to half in about 24 hours at 37.5°C. It has been impossible to determine at which degree this hydrolysis is caused by the cleavage of the choline-ester and salicyl-ester linkage respectively. Both linkages seem to be split non-enzymically, for after 49 hours, when the hydrolysis was still going on, 343 and 122 μl. CO₂ had been evolved

TABLE 9. *Non-Enzymic Hydro-*

Final substrate concentrations in reaction mixture (total volume 2.00 ml.).
Non-enzymic hydrolysis at 37.5° C (at 25.0° C, in brackets) expressed

| Solution | Molarity | pS | μ l. CO ₂ | | Molarity | pS | μ l. CO ₂ | |
|----------|-----------------------|------|--------------------------|---------|-----------------------|------|--------------------------|---------|
| | | | α | 30 min. | | | α | 30 min. |
| No. | ACh chloride | | | | ACh bromide | | | |
| 1 | 1.10×10^{-1} | 0.96 | 4 928 | 32(9.5) | 8.84×10^{-2} | 1.05 | 3 961 | 25.5 |
| 2 | 3.30×10^{-2} | 1.48 | 1 479 | 14(6) | 2.65×10^{-2} | 1.58 | 1 187 | 10 |
| 3 | 1.10×10^{-2} | 1.96 | 493 | 7(4.5) | 8.84×10^{-3} | 2.05 | 396 | 7 |
| 4 | 3.30×10^{-3} | 2.48 | 148 | 5(4) | 2.65×10^{-3} | 2.58 | 118.5 | 5 |
| 5 | 1.10×10^{-3} | 2.96 | 49.5 | 4(3) | 8.84×10^{-4} | 3.05 | 39.5 | 4 |
| 6 | 3.30×10^{-4} | 3.48 | 15 | 3(2) | 2.65×10^{-4} | 3.58 | 12 | 3 |
| No. | BzCh | | | | AAMbZCh | | | |
| 1 | 8.22×10^{-2} | 1.09 | 3 683 | 7.5 | 6.65×10^{-2} | 1.17 | 2 980 | 9 |
| 2 | 2.46×10^{-2} | 1.61 | 1 103 | 3.5 | 2.00×10^{-2} | 1.70 | 896 | 6.5 |
| 3 | 8.22×10^{-3} | 2.09 | 368.5 | 1.5(1) | 6.65×10^{-3} | 2.17 | 298 | 4(2) |
| 4 | 2.46×10^{-3} | 2.61 | 110.5 | 0 | 2.00×10^{-3} | 2.70 | 89.5 | 3 |
| 5 | 8.22×10^{-4} | 3.09 | 37 | 0 | 6.65×10^{-4} | 3.17 | 30 | 2 |
| 6 | 2.46×10^{-4} | 3.61 | 11 | 0 | 2.00×10^{-4} | 3.70 | 9 | 0 |
| No. | CbCh | | | | AAn | | | |
| 1 | 1.09×10^{-1} | 0.96 | 4 883 | 4 | — | — | — | — |
| 2 | 3.29×10^{-2} | 1.48 | 1 474 | 3 | 1.58×10^{-2} | 1.80 | 708 | 5.5 |
| 3 | 1.09×10^{-2} | 1.96 | 488.5 | 2(1) | 5.28×10^{-3} | 2.28 | 236.5 | 3(1) |
| 4 | 3.29×10^{-3} | 2.48 | 147.5 | 2 | 1.58×10^{-3} | 2.80 | 71 | 1.5 |
| 5 | 1.09×10^{-3} | 2.96 | 49 | 1 | 5.28×10^{-4} | 3.28 | 23.5 | 0 |
| 6 | 3.29×10^{-4} | 3.48 | 14.5 | 0 | 1.58×10^{-4} | 3.80 | 7 | 0 |
| No. | ASa | | | | | | | |
| 3 | 1.11×10^{-2} | 1.95 | 497.5 | 7(3) | | | | |

from ASaCh(3) and ASaCh(4) respectively and these values are above the α values (296.5 and 89.5 μ l. respectively) for complete destruction of one ester linkage in ASaCh. Unfortunately, salicylcholine has not been placed at my disposal in sufficient amounts, and therefore only a few preliminary experiments have been carried out with this substance.

Tributyrin was used in the form of emulsions in R₃₀ and these were quite stable at least in lower concentrations. This holds also for the R₃₀ solutions of ethyl acetate. But the 1.11×10^{-2} -M solution of acetylsalicylic acid, used in some experiments as sodium salt, is fairly unstable.

lysis of Substrate Solutions

pS = $-\log$ molar concentration. Total hydrolysis corresponds to α μ l. CO₂ in μ l. CO₂ per 30 min. (mean of 3 to 6 determinations). Cf. Fig. 1.

| Molarity | pS | $\mu\text{l. CO}_2$ | | Molarity | pS | $\mu\text{l. CO}_2$ | |
|-----------------------|------|---------------------|---------|-----------------------|------|---------------------|----------|
| | | α | 30 min. | | | α | 30 min. |
| ACh iodide | | | | McCh | | | |
| 7.32×10^{-2} | 1.13 | 3 279 | 16.5 | 1.02×10^{-1} | 0.99 | 4 569 | 9 |
| 2.20×10^{-2} | 1.66 | 995 | 9 | 3.07×10^{-2} | 1.51 | 1 375 | 3 |
| 7.32×10^{-3} | 2.13 | 328 | 5 | 1.02×10^{-3} | 1.99 | 457 | 1(0) |
| 2.20×10^{-3} | 2.66 | 99.5 | 4 | 3.07×10^{-3} | 2.51 | 137.5 | 0 |
| 7.32×10^{-4} | 3.13 | 33 | 3 | 1.02×10^{-3} | 2.99 | 45.5 | 0 |
| 2.20×10^{-4} | 3.66 | 10 | 2 | 3.07×10^{-4} | 3.51 | 14 | 0 |
| SaCh | | | | ASaCh | | | |
| — | — | — | — | 6.62×10^{-2} | 1.18 | 2 966* | 39(13.5) |
| — | — | — | — | 1.99×10^{-2} | 1.70 | 896 | 13(8) |
| 7.20×10^{-3} | 2.14 | 322.5 | 17 | 6.62×10^{-3} | 2.18 | 296.5 | 7(4.5) |
| 2.16×10^{-3} | 2.67 | 97 | 9 | 1.99×10^{-3} | 2.70 | 89.5 | 5(3.5) |
| 7.20×10^{-4} | 3.14 | 32.5 | 4 | 6.62×10^{-4} | 3.18 | 29.5 | 2.5(2.5) |
| 2.16×10^{-4} | 3.67 | 9.5 | 2 | 1.99×10^{-4} | 3.70 | 9 | 2(2) |
| TB | | | | EA | | | |
| 6.62×10^{-2} | 1.18 | | 1.5(0) | 2.27×10^{-1} | 0.64 | 10 200 | 4 |
| 1.99×10^{-2} | 1.70 | | 0.5(0) | 6.81×10^{-2} | 1.17 | 3 050 | 2.5 |
| 6.62×10^{-3} | 2.18 | | 0(0) | 2.27×10^{-2} | 1.64 | 1 020 | 1(0) |
| 1.99×10^{-3} | 2.70 | | 0(0) | 6.81×10^{-3} | 2.17 | 305 | 0 |
| 6.62×10^{-4} | 3.18 | | 0(0) | 2.27×10^{-3} | 2.64 | 102 | 0 |
| 1.99×10^{-4} | 3.70 | | 0(0) | 6.81×10^{-4} | 3.17 | 30.5 | 0 |

* Calculated for the hydrolysis of one ester linkage.

The possible spontaneous liberation of carbon dioxide from the enzyme preparations has also been examined. In almost every case, no CO₂ was given off. In some few experiments (*e.g.*, guinea-pig liver and kidney), the amounts of CO₂ spontaneously released were 1—3 μ l. in 30 minutes, but these small amounts are quite within the limits of the errors of the method. Therefore the possible amounts of CO₂ spontaneously given off from enzyme preparations have been ignored.

When the enzyme preparations, however, contain carbonic anhydrase, as they do in the case of red blood cells, particular attention must be directed to the action of this enzyme. This action is noticeable immediately after mixing haemolysate with bicarbonate solution and seems to reach an equilibrium after

2—3 minutes. The action of carbonic anhydrase will be discussed more fully below (p. 79) in connection with the interpretation of the course of hydrolysis catalysed by ChE. It has been found that the erythrocytes of all vertebrates investigated, both mammals and fish, contain carbonic anhydrase. It is present also in the blood of *Spirographis* and, in traces, of *Helix*. A very weak activity of carbonic anhydrase was observed with the extracts of liver and brain.

CHAPTER VI

CHOLINESTERASE ACTIVITIES OF BLOOD AND TISSUES FROM VARIOUS ANIMALS COMPARED WITH SOME OTHER ESTERASE ACTIVITIES

A. INTRODUCTION

An enzymic "mapping out" of the kind described in this Chapter is more justifiable than ever. As described above (p. 19), the investigations of recent years have shown that the ACh-hydrolysing enzyme is not to be regarded as a single entity. This is proposed at first by comparing the ChE activities of various tissues. These results invite to do more comprehensive works of the same kind. In consequence, it will be necessary to re-examine the problem of the ACh-ChE system by testing the enzyme activity in the light of these new findings. It has been known for a long time that an enzyme of one species of animal may differ greatly from that of another. In all likelihood, a countless number of enzymes exists, and it would seem logically to suppose that each species has its own equipment of enzymes. No doubt, however, there exist in each enzymic reaction general features which are of the same kind in organisms far removed from each other in the evolutionary scale. This has been shown already, for example, with the enzyme splitting ACh. But it is to be noted, that information concerning the character and the range of distribution of the enzymes found in different groups of animals is far from complete. A complete list of enzymes found in animals, with their range of distribution would, I feel sure, give evidence of a much more regular distribution of separate enzymes than seems to be the case from the investigations performed up till now.

The following list forms the results of an attempt to get information about the distribution and the specificity of the ACh hydrolysing enzyme(s) in different animals. The material has been selected from organisms belonging to the most important classes of the animal kingdom, from the highest group (mammals) to one of the lowest (sea anemones). It may be pointed out that data such as are reported in this Chapter, are not adequate for basing definite conclusions as to the specificity of esterases. The measurements of reaction rates have been carried out under arbitrary standard conditions and the data obtained are simply a basis for further more detailed investigations on the problem of the specificity of cholinesterases.

The esterase activities of blood and tissues have been determined, using choline esters as well as other esters as substrates (Table 6). The interest has been concentrated upon acetylcholine (ACh), remembering that this substance is the only choline ester chemically identifiable in the animal organism and that ACh is the physiological substrate of the specific ChE. The ACh hydrolysing effect has been compared, in the first place, with the activity towards acetyl- β -methylcholine (MeCh) (cf. p. 50 and 110), and benzoylcholine (BzCh). As is related above (p. 50), these two substrates have been proposed for distinguishing the specific ChE from the non-specific one: MeCh is only hydrolysed by the specific enzyme, BzCh by the non-specific one. The inadequacy of this hypothesis has been recognised previously by the present author (AUGUSTINSSON, 1946 b).

The enzymic hydrolysis of the recently synthesised N-methyl- α -aminobenzoylcholine (AAmBzCh), the physiological actions of which have been studied by EULER, EULER and HERRMANN (1945), was tested in some cases, to also the hydrolysis of carbamoylcholine (CbCh), introduced into therapeutics as a substitute for ACh. During the course of the investigations, the interest has been more and more directed to three derivatives of salicylic acid. Unfortunately, too little salicylcholine (SsCh), synthesised for the first time by EULER and his co-workers, was placed at the disposal of the author to permit of more than a few experiments with this substance. An interesting substance in esterase studies is acetylsalicylcholine (ASsCh), also synthesised for the first time by EULER's group (Table 6). This molecule contains two ester linkages, one between acetic acid and the hydroxyl group of the hydroxybenzoic acid, the other between the

carboxyl group of salicylic acid and choline. The enzymic cleavage of this ester has been compared with the enzymic effect upon acetylsalicylic acid (ASa), used as sodium salt, and in some cases with that upon salicylcholine.

From two points of view it may be of interest to investigate whether the same enzyme destroys ACh and acetylcholine (AAn). To begin with, the two primary alcohols choline and aneurine each contain a quaternary N atom. Secondly, it is considered as proved that aneurine also plays an important rôle in the transmission of nerve impulses (p. 5); about 100 times more aneurine is liberated from excited nerves than from non-excited ones and it has been proposed (p. 18) that aneurine in the form of its acetic-acid ester might play a similar part to that of ACh in nervous activity.

The main purpose of this investigation has been to study the specificities of the choline-ester hydrolysing enzymes. Because of that it was important to correlate the results obtained with choline esters with those obtained with ordinary esters. Tributyrin (TB) and ethyl acetate (EA) have been chosen as substrates for ordinary esterases (lipases), which hydrolyse esters of fatty acids and glycerol or simple alcohols respectively.

Regarding the selection of enzyme preparations, the following points are to be noted. The characteristics of the ChE activities of blood from various animal species have been seen to vary greatly (p. 6). Accordingly, the present investigation was performed with blood from seven vertebrate species (mammals, fowl, fish) and two invertebrate ones (snail, worm). In each case, serum (plasma) and the haemolysate of the erythrocytes have been investigated separately. *Helix* blood has been shown previously to be very ChE active with interesting properties (p. 21). The blood of *Spirographis* has a special biochemical interest, not concerned here, in containing the respiratory pigment chlorocruorin; this blood has not been used previously in ChE studies.

Next to blood, nervous and muscle tissues have been used most frequently in the determinations of ChE activity (p. 8). In most of the present investigations, dog brain has been used; a few experiments have been carried out with brain from bear and elephant, two species not very frequently found in scientific papers of this kind. The investigation deals also with the central nervous systems of two bony fish (*Gadus*, *Labrus*), two sharks (*Scyllium*, *Squalus*), the common ray, and one of the lowest vertebrates known (*Myxine*). Also the muscles of these species were

used in some cases. A hypothesis has prevailed for a long time that the blood esterase is formed in the liver, and previous studies offer proofs that this holds for the serum ChE (p. 10). The activity of the hepatic ChE is therefore of great interest to and we will compare the properties of the liver enzyme with those of the blood enzyme. Preliminary experiments, however, have shown that the liver esterases vary greatly in their affinities for different esters. Because of that, liver preparations have been made from various species, both mammals, fowl, and fish. Attention has been directed especially to the guinea-pig liver. The esterase activity of this organ has been said to show peculiar properties, not found elsewhere (p. 20). The present author has studied the activities of the kidneys of cow and guinea pig. Also the smaller intestine of guinea pig has been used.

Among invertebrate tissues, those of the cuttlefish have been studied previously by the author (p. 21). More extensive investigations will be described in this paper with the same material. The ink of *Sepia* has not been used previously in ChE studies. This is also the case with some parts of the rare *Echinozephyra*. The last animal studied is an anemone (*Negadia*) which is one of the most animals in the evolutionary scale with a well-developed nervous system. Finally, the ChE activity has been determined in the developing sea-urchin eggs (*Paracentrotus*).

A more detailed account of the ChE activities generally compared with other esterase activities is given below and summarised in Tables 10 and 16. The data in these Tables represent $\mu\text{l. CO}_2$ evolved during 30 min. for 0.5 ml. of sample, and calculated for 100 $\mu\text{l. blood (plasma)}$ and 100 $\mu\text{l. tissue}$, respectively. The values represent in most cases the initial values of the activity-time curves. When the reaction rate fell rapidly, and therefore it was impossible to state this, the initial values of the enzyme activities were expressed as the actual amount of CO_2 evolved during 30 min. minus the value of non-enzymatic hydrolysis. Such data are placed in parentheses (cf. p. 94). The table also contains also the results of some purification experiments of ChE.

B. Blood

1. Blood of Vertebrates

a) Serum (Plasma)

Man. Human blood plasma splits ACh at a high rate (Table 10). The enzyme is of the non-specific type, according to the

TABLE 10. *Enzymic Hydrolysis of Certain Esters by Blood from Different Animals*

$\mu\text{l. CO}_2$ evolved during 30 min. = b_{30} , calculated for 100 $\mu\text{l.}$ blood (plasma).
The substrate concentrations are those of the solutions No. 3 (Table 9).

| Species | ACh | MeCh | BzCh | AAmBzCh | CbCh | SaCh | ASaCh | ASa | AAm | TB | EA |
|--------------------|-----|------|------|---------|------|------|-------|-----|-----|------|----|
| Man | | | | | | | | | | | |
| Plasma | 137 | 4 | 73 | 3 | 0 | — | 107 | 5 | 21 | 65 | 3 |
| Erythrocytes .. | 167 | (78) | 3 | 1 | 0 | — | (98) | 5 | 7 | 23 | 1 |
| Horse | | | | | | | | | | | |
| Plasma | 353 | 11 | 161 | 2 | 1 | 58 | 290 | 8 | 19 | 160 | 12 |
| Erythrocytes .. | 30 | 15 | 4 | 0 | 0 | — | 30 | 3 | 0 | 15 | 2 |
| Cow | | | | | | | | | | | |
| Plasma | 5 | 3 | 1 | 0 | 0 | 0 | 12 | 2 | 2 | 13 | 0 |
| Erythrocytes .. | 163 | 70 | 4 | 0 | 0 | 0 | 121 | 3 | 9 | 28 | 2 |
| Guinea pig | | | | | | | | | | | |
| Plasma | 84 | 11 | 40 | 1 | 0 | 9 | 68 | 15 | 25 | (57) | 5 |
| Erythrocytes .. | 60 | 46 | 2 | — | — | — | 42 | 8 | 16 | 23 | 1 |
| Fowl | | | | | | | | | | | |
| Plasma | 28 | 11 | 3 | 0 | 0 | — | (19) | 2 | 10 | 23 | 2 |
| Erythrocytes .. | 0 | 1 | 1 | 2 | 0 | — | 5 | 1 | — | 3 | 1 |
| Labrus | | | | | | | | | | | |
| Plasma | 5 | 2 | 1 | — | — | — | 17 | 2 | 7 | 3 | — |
| Erythrocytes .. | 0 | 0 | 1 | — | — | — | — | — | — | 0 | — |
| Scyllium | | | | | | | | | | | |
| Plasma | 9 | 5 | 1 | 0 | 0 | — | 12 | 1 | 2 | 0 | 0 |
| Erythrocytes .. | 0 | 0 | 0 | — | — | — | 0 | 2 | 0 | 0 | 0 |
| Helix | 582 | 157 | 16 | 0 | 0 | 0 | 112 | 41 | 188 | 25 | 15 |
| Spirographis | 5 | 4 | 0 | — | — | — | 270 | 254 | 12 | 15 | 0 |

definition of MENDEL's group (p. 20); BzCh is hydrolysed, but not MeCh. ASaCh is split, but not ASa. Therefore, it may be supposed that the plasma hydrolyses the choline-ester linkage only in ASaCh, and most probably the same enzyme is responsible for the hydrolysis of ASaCh and BzCh. AAmBzCh is not split. The lack of hydrolysis of CbCh is also found with almost all other enzyme preparations. AAm is split, and most probably a ChE takes part in this reaction. In all probability, this is also true in the hydrolysis of TB (cf. below, horse plasma).

Horse. Most studies by the author on the serum ChE, both crude and purified preparations, have been performed with horse serum. It splits ACh at a higher rate than does human plasma. According to MENDEL's hypothesis, horse plasma contains a non-specific ChE (BzCh hydrolysed) and in very low concentration a specific one (MeCh hydrolysed at a low rate) (p. 20). The activity towards ASaCh is high and also SaCh is

TABLE 11. *Comparison of the ChE Activity of Horse Plasma and a Purified Plasma Enzyme Preparation*

| Substrate | Plasma | | Purified enzyme
<i>Cf</i> \approx 3 000 | |
|-------------|------------------------|----------|--|----------|
| | <i>b</i> ₃₀ | % of ACh | <i>b</i> ₃₀ | % of ACh |
| ACh | 353 | 100 | 320 | 100 |
| MeCh | 11 | 3.1 | 11 | 3.4 |
| BzCh | 161 | 45.6 | 141 | 44.1 |
| ACh | 133 | 100 | 320 | 100 |
| SaCh | 23 | 17.3 | 53 | 16.6 |
| ASaCh | 113 | 85.0 | 278 | 86.9 |
| TB | 61 | 45.8 | 149 | 46.6 |

split at a relatively high rate. In conformity to human plasma, the horse plasma splits TB. Confirming previous results of the author, AAn is hydrolysed.

Several experiments have been carried out with purified horse-serum ChE, the affinities of which for various esters have been studied and compared with those obtained with crude preparations. The method of purification is described above (p. 42).

In a previous communication (AUGUSTINSSON, 1944), the author reported that the hydrolysis of ACh and AAn by horse serum must be attributed to the same enzyme. This was shown in two ways. The hydrolysis by serum and purified enzyme preparation was compared by means of the ratio between the amounts of CO₂ evolved using ACh and AAn as substrates. A constant quotient was obtained, indicating that the same enzyme is responsible for the two reactions. Moreover, it was shown by means of electrophoresis experiments that "the two enzymes" move parallel in the electric field.

Further experiments of the same kind with horse plasma have been performed with other esters. As the purification proceeds, the rates of hydrolysis have been compared. The results appear in Table 11. The activities towards ACh, MeCh, BzCh, SaCh, ASaCh, and TB run parallel, and because of that, it may be inferred that the same enzyme is responsible for the hydrolysis of all these esters. As regards the hydrolysis of TB, this result confirms previous findings by other authors (p. 19). It is inadvisable to speak about separate esterases in blood plasma responsible for the hydrolysis of ACh and TB, and the esterase is really a non-specific ChE.

Cow. In contradistinction to the erythrocytes of cow (p. 60) and to the plasma of man and horse, the cow plasma is practically inactive towards choline esters used in this investigation. TB is also hydrolysed at a very low rate.

Guinea pig. The ChE activity of guinea-pig plasma is lower than that of human and horse plasma, but nevertheless considerable. The characteristics of the enzyme seem to correlate with those of the non-specific type. The rate of the BzCh hydrolysis is about 50 per cent of the ACh hydrolysis. This is also true for the human and horse plasma. MeCh is split to the same extent as by the horse plasma. ASaCh and TB are hydrolysed at relatively high rates. The activity towards AAn is the same as is found for other plasma preparations.

Fowl. The esterase activity of cock plasma is quite low. It differs from other plasma activities investigated in the relatively higher activity towards MeCh compared with that towards BzCh. The ChE of cock plasma therefore seems to be of the specific type (p. 107). This plasma hydrolyses ASaCh, TB, and AAn at low rates.

Labrus. The blood plasma of this bony fish is not very ChE active. ASaCh seems to be the only ester which is split at a rate worth mentioning, but really it is very low.

Scyllium. Low ChE activity has also been found in the plasma of the dogfish shark. It seems to contain a specific ChE in low concentration (p. 108).

To sum up the results obtained with blood plasma, the highest activity towards ACh is possessed by horse plasma. The plasma of man, guinea pig, and fowl come next; the activity of the fowl plasma is lower than one tenth of that of the horse plasma. Remarkably low activity was found with cow and fish plasma. According to the definition of MENDEL and his co-workers, the enzyme is of the non-specific type in the plasma of man, horse, and guinea pig; a specific ChE seems to be present in the plasma of fowl and, in very low concentrations, of fish. The hydrolysis of BzCh, SaCh, and ASaCh parallel that of ACh and this is also true for the cleavage of TB. It is a working hypothesis for the extension of the specificity problem of ChE that the same enzyme in blood plasma, in most cases, splits ACh, BzCh, SaCh, ASaCh, and TB (and AAn). When the plasma hydrolyses ACh at a high rate, this rate is higher than those for other esters. It is therefore justifiable in this case to speak about a ChE. But the esterase is not specific towards choline esters, it also splits TB (and AAn).

b) *Erythrocytes*

As was expected from previous findings of various authors (p. 19), the ChE activity of the blood cells differs to a great extent from that of the plasma. The results were according to the following. The b_{50} values listed in Table 10 are calculated for 100 μ l. haemolysate, prepared by adding distilled water to the washed blood cells to blood volume.

Man. Human erythrocytes are highly active towards ACh. MeCh, but not BzCh, is hydrolysed and thus the enzyme is a specific ChE. The shape of the reaction curve for MeCh is not the same as that for ACh; the hydrolysis of MeCh seems to follow a first-order reaction, that of ACh a zero-order one. Such differences between the ACh and MeCh hydrolysis have been found with other material and are discussed more fully in Chapter VII. The reaction curves of MeCh resemble those of ASaCh, which is split at about the same rate as MeCh. The activities towards other esters are considerably lower.

Horse. As distinct from the erythrocyte ChE of human blood, that of horse blood hydrolyses ACh and other choline esters at a much lower rate than the corresponding plasma. The enzyme seems to be of the specific type. ASaCh is split at the same rate as ACh, TB as MeCh.

The ChE activities of whole blood and haemolysed blood have been compared, so also the activities of unhaemolysed and haemolysed blood cells. Table 12 lists the results of such an experiment. Blood, haemolysed with distilled water, was found to have the same activity as whole blood diluted with 0.9 per cent NaCl solution to give the same blood concentration. In another experiment washed cells were suspended in the saline solution and the ChE activity of the suspension determined. The same value was obtained with this preparation as with another prepared from the same amount of blood cells haemolysed with distilled water of volume equal to that of the saline solution.

Experiments have been carried out in purifying the ChE of red blood cells. The method is described above (p. 43), the results reported next and summarised in Tables 13 and 14 and Fig. 2. The principle of the first stage of this method is based on the flocculation of the cell membrane, to which the enzyme is bound. This procedure has been carried out successfully in lowering the pH value of the haemolysate to 6.5—6.6 (horse blood). In order

TABLE 12. *ChE Activities of Whole and Haemolysed Blood and of Unhaemolysed and Haemolysed Erythrocytes*

Blood taken up in heparin. Substrate ACh(3).

| Blood | Enzyme preparation | b_{30} |
|-------|---------------------------------|----------|
| Horse | Whole blood | 393 |
| | Haemolysed blood | 393 |
| Cow | Erythrocytes in 0.9 % NaCl..... | 159 |
| | Erythrocytes, haemolysate | 156 |

to find this most suitable pH for the precipitation of the enzyme, the pH values were lowered gradually by adding HCl, and the ChE activities of the precipitates, suspended in water, and of the centrifugates measured. The activity values were compared with those ($b_{30}/2$) obtained with the suspensions after the adjustment of pH and before centrifugating. The fact is that the enzyme is gradually inactivated when pH is lowered, in agreement with previous findings by the author (AUGUSTINSSON, 1944). The degree of this inactivation is also found in Table 13 and Fig. 2, and is calculated as “% of b_{30} (1)”; b_{30} (1) is the activity of the haemolysate of the same concentration as that of the reaction mixture after the treatment with HCl. The pH value of the original haemolysate of horse blood cells was 7.43. In the flocculation of the cell membrane, the ChE activity follows the “ghosts”.

TABLE 13. *Precipitation of ChE from Haemolysate*

| Blood | Substrate
(sols. No. 3) | Haemolysate
ml. | 0.1-N HCl
ml. | pH | ChE activity | | | | | | |
|------------|----------------------------|--------------------|------------------|------|-----------------------------|-------------------------------|----------------------------|--------------|------------------------|-------------|------------------------|
| | | | | | Haem.
+ H ₂ O | Centrifugate
+ precipitate | | Centrifugate | | Precipitate | |
| | | | | | | <i>b</i> ₃₀ (1) | <i>b</i> ₃₀ (2) | % of
(1) | <i>b</i> ₃₀ | % of
(2) | <i>b</i> ₃₀ |
| Horse | ACh | 20.0 | 1.36 | 6.94 | 53.5 | 53.5 | 100 | 34 | 63.5 | 17 | 32 |
| » | » | 20.0 | 1.80 | 6.75 | 53.5 | 50 | 93.5 | 7 | 14 | 42 | 84 |
| » | » | 20.0 | 2.15 | 6.54 | 54 | 47 | 87 | 2 | 4.5 | 44.5 | 95 |
| » | » | 20.0 | 2.52 | 6.37 | 51 | 41 | 80.5 | 6 | 14.5 | 36 | 88 |
| » | » | 20.0 | 2.90 | 6.20 | 50 | 39 | 78 | 7 | 18 | 30 | 77 |
| Horse | MeCh | 20.0 | 2.52 | 6.37 | — | 33.5 | — | 9.5 | 28.5 | 27 | 80.5 |
| » | TB | 20.0 | 1.80 | 6.75 | — | 44 | — | 32 | 73 | 14 | 32 |
| Cow | ACh | 195.0 | 19.10 | 6.51 | 290 | 238 | 82 | 236 | 99 | 4 | 1.5 |
| » | » | — | — | 5.53 | — | 210 | — | 2 | 1 | 204 | 97 |
| Guinea pig | ACh | 10.0 | 1.00 | 6.41 | 119 | 105 | 88.5 | 2 | 2 | 109 | 100 |

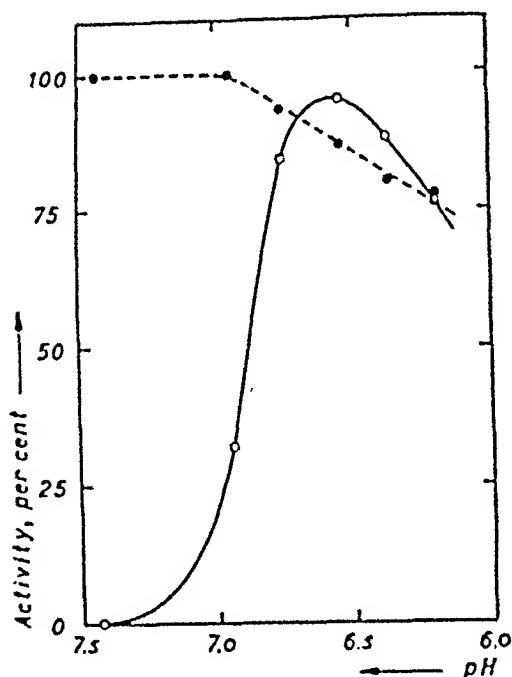


Fig. 2. Precipitation of ChE from haemolysate of horse blood cells. Broken line refers to inactivation of ChE by lowering of pH, unbroken line to per cent of ChE in precipitate as function of pH. Data from Table 13.

At pH 6.5—6.6 a very distinct pH range was obtained at which the separation is almost complete. This interval does not seem to be a general one for all kinds of erythrocyte ChE. In the case of cow blood, for instance, the optimum precipitation was found to occur at a lower pH value (≈ 5.5).

After the completion of the experimental work, the author was informed of the results of PALÉUS (1947) who found that the erythrocyte ChE (man, dog) is attached to the stroma of the cells. This was shown by a method similar to that described above.

The enzyme activities of the precipitates towards other esters have also been measured in some cases. Table 13 shows that the activity towards MeCh parallels that towards ACh. With TB, however, such a parallelism was not obtained. At pH 6.75, the ChE activity was found to 84 per cent in the precipitate, the tributyrinase activity to 32 per cent only. This observation provides further support for the supposition that in the red blood cells of horse two different enzymes are responsible for the hydrolysis of ACh and TB.

It has been a difficult task to elute the enzyme from the precipitate (Table 14). By shaking the suspension of a ChE precipitate with a 10 per cent ammonium-sulphate solution, practically no enzyme went into solution. Nor did a phosphate buffer of pH

TABLE 14. *Attempts by Elution to Separate ChE from Flocculated Stroma*
Horse erythrocytes and ACh(3).

| Procedure | b_{30} | | | |
|---|------------------------------|------------------|--------------|-----|
| | Original
prepara-
tion | Precipi-
tate | Centrifugate | |
| | | | b_{30} | % |
| Precipitation at pH 6.75. 1.0 ml. precipitate in H_2O + 1.0 ml. $(NH_4)_2SO_4$ (10 %). Shaking one hour. Centrifugating | 25 | 21 | 1 | 0 |
| Precipitation at pH 6.47. Washing with H_2O four times. Washing with phosphate buffer (pH 6.60). Shaking one hour. Centrifugating | 23 | 22 | 0 | 0 |
| Precipitation at pH 6.47. Washing with H_2O twice and with phosphate buffer twice. 8.0 ml. suspension of the same volumes of precipitate and H_2O + 2.0 ml. H_2O + 2.0 ml. bicarbonate solution of lysolecithin (0.1 %). Shaking one hour. Centrifugating | 66 | 0 | 70 | 100 |

6.60 elute the enzyme; three times washing with this buffer did not alter the ChE activity of the precipitate. When, however, the precipitate was treated with a 0.1 per cent solution of lysolecithin, a centrifugate was obtained which contained the ChE activity to 100 per cent. In this process about half of the precipitate went into solution. Lysolecithin does not affect the enzyme activity (Table 15).

Cow. In contrast to the cow plasma which does not contain ChE, the correspondent erythrocytes are very active towards ACh. The enzyme is a specific ChE, for it hydrolyses MeCh, but not BzCh. As with other blood cell preparations no activities have been detected towards AAmBzCh and CbCh. TB is split at low rate, ASaCh at high. SaCh is not attacked at all, ASa and AAn very little.

TABLE 15. *Effect of Lysolecithin on ChE*
Substrate ACh(3).

| Three volumes of | b_{30} | |
|-------------------------------------|--------------------|----------------------|
| | One volume of | |
| | $NaHCO_3$ (1.26 %) | lysolecithin (0.1 %) |
| Serum ChE (horse), purified | 209 | 205 |
| Erythrocytes (horse), haemolysate . | 50 | 53 |

The ChE of cow erythrocytes has been precipitated in the same way as the enzyme of horse blood cells. In contradistinction to the latter enzyme, the cow enzyme is flocculated at a lower pH value or about 5.5 (Table 13).

Guinea pig. The esterase activity of guinea-pig erythrocytes shows the same properties as those found for other mammalian species. The activity is not very high, somewhat higher than was found for the horse blood. The ChE activity in its main features shows the characteristics of a specific ChE. The enzyme has been precipitated from haemolysate. In one experiment (Table 13), the ChE was completely precipitated together with the stroma at pH 6.41.

Fowl erythrocytes as well as those of *fish* do not contain any esterases.

Summing up, the erythrocyte-ChE activity of blood decreases according to the following series: man, cow, guinea pig, horse. In all ChE-active erythrocytes tested the enzyme is a specific ChE which splits MeCh, but not BzCh. No erythrocyte preparation was found to hydrolyse BzCh. The same series seems to hold for the hydrolysis of ASaCh, the rate of which is lower than in the case of plasma (except cow). Most probably, the same enzyme splits ACh, MeCh, and ASaCh. TB is hydrolysed at a low rate by an enzyme not identical with ChE. Other esters are not or very little split.

ChE is bound to the cell membrane and can be precipitated together with the stroma by adding hydrochloric acid to pH 6.5 (horse blood) or 5.5 (cow blood). The enzyme goes into solution by treating the precipitate with lysolecithin.

2. Blood of Invertebrates

a) *Helix pomatia*

Some results obtained with the blood of *Helix pomatia* have been published recently (AUGUSTINSSON, 1946 c). This invertebrate blood is several times more active towards ACh than the blood of vertebrates (Table 10). The enzyme resembles the erythrocyte ChE in hydrolysing MeCh at a higher rate than BzCh. But the affinity of MeCh for the snail blood ChE is only about 28 per cent of the affinity of ACh for the same enzyme. The affinity of MeCh for the erythrocyte ChE is about 50 per cent of that of ACh. ASaCh is hydrolysed and it will be shown later on in this paper

(p. 153) that the ACh-splitting enzyme catalyses this reaction. At a low rate ASa is enzymically destroyed by the *Helix* blood. The high rate of hydrolysis of AAn is especially noticeable; the b_{30} value of this reaction is the highest ever found with AAn as substrate. TB is split, but the reaction velocity is relatively very low.

As regards experiments performed in order to purify the ChE of *Helix* blood, the following points should be noted. The blood loses some of its activity by dialysis. In a typical experiment the ChE activity of a blood sample (diluted 1 : 40) was b_{30} 36; after dialysis the activity was reduced to 25, calculated for the same blood concentration. The enzyme was reactivated by adding the dialysate (b_{30} 38). It was precipitated by adding ammonium sulphate to 55 per cent saturation. The yield of this procedure was 90—100 per cent. The horse plasma ChE is precipitated at a much higher salt concentration (p. 43).

The b_{30} value obtained in an experiment was 171 for the original dialysed blood solution. After precipitation the centrifugate showed b_{30} 5; b_{30} of the dialysed solution of the precipitate in the same volume of distilled water as the original volume was 155. In such procedures the total protein concentration decreased about 40 per cent. The protein concentrations of the solutions, the ChE activities of which have been reported, were 0.66 per cent for the original one and 0.40 per cent for the solution of the precipitate. Some preliminary experiments have also been carried out in order to purify the enzyme further. Thus the enzyme was adsorbed completely on infusorial earth from a solution of the sulphate precipitate. After treating 10 ml. of such a solution (b_{30} 504) with 0.1 g. and 0.3 g. infusorial earth, the centrifugates gave b_{30} 116 and 0 respectively. A suitable medium, however, has not yet been found by which the enzyme can be eluted from the earth.

b) *Spirographis Spallanzani*

The esterase activity of the blood of this polychaete worm has been studied at 25.0°C, in contrast to the studies just described (37.5°C). The activity towards choline esters is very low and it is hard to say from these preliminary experiments, whether the activity corresponds to a specific or a non-specific ChE. The blood hydrolyses MeCh at about the same low rate as ACh, and it does not split BzCh. AAn is attacked a little and so also TB; EA, on the other hand, is quite unaffected.

The great ability of the blood to hydrolyse salicylic esters is surprising; the reaction rates in these cases are 50 times higher than the rate when ACh is used as substrate. Properties of the same kind have been found for the esterases of other material

from mammals and will be discussed below (p. 68). It is supposed that the blood of *Spirographis* contains a "salicylesterase" which hydrolyses ASa. Proofs have been presented that in the case of ASaCh it is the linkage between the acetyl group and the phenol group of the salicylic rest which is destroyed, that is, the same linkage as in ASa. More details about the esterase activity of *Spirographis* blood are found in a recent paper (AUGUSTINSSON, 1947).

C. TISSUES

1. Tissues of Vertebrates

a) Brain

Dog. As is previously found by various authors, the brain ChE hydrolyses MeCh at a relatively high rate. In contradistinction to these previous results, the present author has observed that BzCh is also split, but at a very low rate (Table 16). Hence, according to the accepted hypothesis, the brain, as all other nerve tissues, contains a specific ChE. ASaCh is split at about the same rate as ACh, so also TB. Lower rates of hydrolysis were found with ASa, AAn, and EA as substrates.

Preliminary experiments have been performed in order to purify the enzyme of dog brain. The experiments, however, have given no useful results. The enzyme is not precipitated in good yield by ammonium sulphate at 50 per cent saturation of the original extracts.

Labrus. The brain of this bony fish contains a specific ChE in high concentration. The hydrolysis of ASaCh was also considerable. AAn is split at a relatively high rate. The determinations were carried out at 25.0°C.

Scyllium. In comparison with the brain of *Labrus*, the shark brain (*Scyllium* as well as *Squalus* (Table 17)) has a low ChE activity, the characteristics of which are of the same kind as those of the activity of bony-fish brain. The rates of hydrolysis were lower in all cases of substrates used.

Brains of other vertebrates. The ChE activities of other vertebrate brains have also been studied (Table 17). These include bear, elephant, cod, *Squalus*, ray, and hagfish, the brains of which have been used in studies on the enzymic hydrolysis of ACh, MeCh, and BzCh. Confirming previous results, nucleus caudatus has a high con-

TABLE 16. *Enzymic Hydrolysis of Certain Esters by Tissues from Different Animals*

μ l. CO₂ evolved during 30 min. = b_{30} , calculated for 100 mg. tissue. The substrate concentrations are those of the solutions No. 3 (Table 9).

| Tissues-Species | ACh | MeCh | BzCh | AAmBzCh | ObCh | SaCh | ASaCh | ASa | AAm | TB | EA |
|-----------------------|-----|------|------|---------|------|------|-------|-------|------|-------|------|
| Brain (cf. Table 17) | | | | | | | | | | | |
| Dog | 59 | 27 | 15 | 3 | 3 | — | 64 | 13 | 7 | 59 | 10 |
| <i>Labrus</i> | 800 | 92 | 9 | — | — | — | 268 | 21 | 74 | 31 | — |
| <i>Scyllium</i> | 43 | 16 | 4 | 0 | 0 | — | 36 | 14 | 1 | 2 | 0 |
| Muscle (cf. Table 18) | | | | | | | | | | | |
| Dog | 14 | 9 | 23 | 2 | 2 | — | 37 | 16 | 20 | 172 | 32 |
| <i>Labrus</i> | 46 | 21 | 7 | — | — | — | 18 | 6 | 59 | 20 | — |
| Liver (cf. Table 18) | | | | | | | | | | | |
| Cow | 25 | 15 | 9 | 5 | — | — | 203 | 44 | 11 | (316) | 20 |
| Guinea pig | 13 | 9 | 296 | 10 | 21 | 38 | (266) | 273 | 34 | (376) | 23 |
| Fowl | 296 | 114 | 48 | 11 | 38 | — | (248) | 60 | — | (307) | (67) |
| <i>Labrus</i> | 149 | 49 | 10 | — | — | — | 114 | 70 | 103 | 120 | — |
| <i>Scyllium</i> | 7 | 5 | 4 | — | — | — | 40 | 14 | 9 | 32 | 15 |
| Kidney | | | | | | | | | | | |
| Cow | 7 | 7 | 25 | — | — | — | 97 | 23 | 11 | (436) | 23 |
| Guinea pig | 10 | 12 | 23 | 5 | 6 | 1 | 120 | (274) | — | (164) | 9 |
| Intestine | | | | | | | | | | | |
| Guinea pig | 85 | 30 | 29 | — | 4 | 8 | 131 | 37 | (35) | 208 | 16 |
| <i>Helix</i> | | | | | | | | | | | |
| Dart sac | 906 | 70 | 37 | 0 | 1 | 0 | 336 | 18 | 48 | 107 | 10 |
| <i>Sepia</i> | | | | | | | | | | | |
| "Liver" | 120 | 15 | 7 | 0 | 0 | — | 69 | 38 | 12 | 92 | 14 |
| <i>Maia</i> | | | | | | | | | | | |
| Muscle | 26 | 12 | 10 | — | — | — | 2 | 2 | 6 | 0 | 22 |
| <i>Balanoglossus</i> | | | | | | | | | | | |
| Proboscis | 34 | 22 | 0 | — | — | — | 38 | — | 0 | 15 | — |
| Collar | 32 | 16 | 0 | — | — | — | 30 | 0 | 0 | 64 | 0 |
| "Liver-sacs" | 10 | 6 | 2 | — | — | — | 106 | 12 | 4 | 49 | 103 |
| <i>Sagartia</i> | | | | | | | | | | | |
| Parts with nerve net | 11 | 3 | 8 | — | — | — | 110 | 29 | 7 | 107 | — |
| <i>Paracentrotus</i> | | | | | | | | | | | |
| Larvae (Plutei) | 560 | 139 | 4 | — | — | — | 256 | 37 | 21 | 74 | — |

centration of ChE, cortex a low one, shown here in experiments with the brain of bear. All brain esterases show essentially the same pattern. ACh is split at a high rate; the highest esterase concentration was found in the cod brain. The brain of the common ray seems to be more active than the shark brain. Low ChE activity is possessed by the hagfish brain which is biologically more primitive than other vertebrate brains.

Summing up, brain esterases show the same properties in their abilities to hydrolyse various esters. The ability to split ACh at a higher rate than other esters is an important feature of the

TABLE 17. *Enzymic Hydrolysis of ACh, MeCh, and BzCh by Brain Extracts of Different Vertebrates*

100 mg. tissue. Substrate solutions No. 3.

| Species | ACh | MeCh | BzCh |
|----------------------------|-----|------|------|
| Bear | | | |
| Nucleus caudatus | 900 | 570 | 66 |
| Cortex | 37 | 20 | 0 |
| Elephant | 137 | 98 | 7 |
| <i>Gadus</i> | 383 | 105 | 5 |
| <i>Raja</i> | 109 | 62 | 13 |
| <i>Squalus</i> | 28 | 11 | 2 |
| <i>Myxine</i> | 40 | 40 | — |

brain esterase, because of the significant part that is played by ACh in the nervous system, and because ACh is the physiological substrate of the specific ChE. Brain is indeed the only organ used in this investigation that contains a specific ChE, whatever the vertebrate may be. It is said that the brain tissue does not split BzCh. As is seen in Table 16 and 17, the activity towards this ester is lower than towards MeCh, but brain extracts do split BzCh even though at a very low rate. Whether this reaction is to be ascribed to the action of the specific ChE has been hard to find out.

When ASaCh has been used as substrate, the reaction velocities have always been higher than in the case of MeCh. AAmBzCh and CbCh are not split by brain tissue. The rates of hydrolysis decrease generally according to the following series: ACh, ASaCh, MeCh, TB, ASa, BzCh, AAn, EA.

b) Muscle

The ChE activity of striated *dog* muscles is low and, most probably, due to a specific ChE (Table 16). As pointed out above (p. 8), previous investigations have stated that ChE, the concentration of which is surprisingly low in striated muscles, is concentrated at the nerve endings. The relatively high rate of the hydrolysis of BzCh by dog muscle is most probably due to the action of an esterase very active towards TB. Relatively high activities were found towards ASaCh and AAn.

The fish muscles have a stronger ChE activity than most mammalian muscles (Table 18). This possibly is in agreement with a previous statement that the ChE concentration in the

TABLE 18. *Enzymic Hydrolysis of ACh, MeCh, and BzCh by Fish Muscle and Liver Extracts*

100 mg. tissue. Substrate solutions No. 3. The values in brackets refer to heart muscle.

| Species | Muscle | | | Liver | | |
|----------------------|--------|--------|------|-------|------|------|
| | ACh | MeCh | BzCh | ACh | MeCh | BzCh |
| <i>Gadus</i> | 70(91) | 26(34) | 9 | 253 | 85 | 34 |
| <i>Raja</i> | 73(10) | 22(10) | 9(0) | 6 | 2 | 1 |
| <i>Squalus</i> | 49(28) | 22 (6) | 2 | 13 | 7 | 0 |
| <i>Myxine</i> | 109 | 32 | 5 | 4 | 2 | 0 |

muscles of animals that move rapidly is higher than in those organisms which are less active (p. 9). The hagfish muscles have a higher ChE activity than the bony-fish muscles. In all these cases the enzyme seems to possess the characteristics of a specific ChE.

In some preliminary experiments the ChE activities of heart muscles from fish have been measured (Table 18, in brackets). The enzyme seems to be a specific ChE which in low concentration occurs in the heart of Elasmobranchs. As shown in Table 10 the blood of these animals is also very poor in ChE.

c) *Liver*

Cow. Cow liver contains ChE in low concentration. The enzyme is a specific ChE, hydrolysing MeCh at a higher rate than BzCh. ASaCh is split at a very high rate and the activity towards ASa is higher than towards choline esters. Most probably the hydrolysis of TB is catalysed by an esterase separated from the ChE.

Guinea pig. The ChE activity of guinea-pig liver differs greatly from other liver-esterase activities and from most other esterases investigated. During the experiments on the ChE activity of this liver and when the data obtained (1945, unpublished) had shown that BzCh is hydrolysed at a rate which is several hundred per cent higher than in the case of ACh, a communication was published by SAWYER (1945), who assumed that a "benzoylcholine esterase" is present in high concentration in the guinea-pig liver, not concerned with the ACh hydrolysis. The existence of such an esterase has also been discussed later on by BLASCHKO, CHOU and WAJDA (1947 a, b).

The liver of guinea pig hydrolyses ACh at a low rate. TB is

split at high rate and so also ASa¹ and ASaCh. The activities towards SaCh and AAn are not very high. At low rate, the liver splits AAmBzCh and CbCh.

ASaCh contains in its molecule two ester linkages. In experiments with this substrate, it has always been of interest to find out whether one or both and which of the linkages are hydrolysed. In order to get an answer to the first question as regards the hydrolysis of ASaCh by guinea-pig liver, the hydrolysis was allowed to proceed for a long time. Simultaneously, the same experiment was performed in the presence of physostigmine which, in the concentration of 3.63×10^{-6} M, has been shown to inhibit the hydrolysis of ASaCh and BzCh at about 21 and 17 per cent respectively (Table 33). The hydrolysis of TB, on the other hand, is not inhibited at all. The action of physostigmine on ChE will be discussed more fully below (p. 142). It is seen in Fig. 30 (p. 143) that the hydrolysis of ASaCh proceeded after the escape of the theoretical amount of CO₂ (296.5 μ l.), calculated for the cleavage of one of the ester linkages in ASaCh. The reaction curve shows that both linkages are split, and they are so at the same time.

Because of that the author is not inclined to suppose that the guinea-pig liver contains a "benzoylcholine esterase", hydrolysing BzCh at a higher rate than any other ester. It will be shown later on in this paper that this esterase has nothing in common with a ChE. On the contrary, the same enzyme is likely to split, in addition to BzCh, SaCh and the choline-ester linkage in ASaCh. Such an esterase may probably be specific against the esters of aromatic acids. In addition the guinea-pig liver seems to contain an esterase (a "salicylesterase"?) hydrolysing ASa and the corresponding linkage in ASaCh, that is, an esterase of the same type as was detected in *Spirographis* blood (p. 62) and in the kidney and intestine of guinea pig (p. 68).

Fowl. The ChE concentration in cock liver is high and the enzyme has the characteristics of a specific ChE. The liver hydrolyses ASaCh at a high rate and so also TB. ASa and EA are also split, but at a lower rate.

Fish. *Labrus* liver contains a specific ChE in high concentration and this is also true for cod liver (Table 18). ASaCh, AAn, and ASa are also split at high rates. The ChE activities of the livers

¹ In extracts of guinea-pig liver and kidney, VANDELLI and SCALTRITI (1943) have also found an enzyme, distinct from ChE, which hydrolyses acetylsalicylic acid. (Only a short abstract, 1947, of this work has been available.)

from shark, ray, and hagfish are very low; they all contain a specific ChE in low concentration.

It is obvious from the results reported above that the esterase activities of liver differ to a great extent from animal to animal. The concentration is high in some species (cock, bony fish), low in others (cow, shark). When the liver esterases had high affinities for ACh, they showed mostly the characteristics of a specific ChE. The enzyme(s) of guinea-pig liver split BzCh at a remarkably high rate, but it (they) has (have) also a high affinity for ASa and ASaCh (and SaCh). It is supposed that this tissue contains an esterase specific against esters of aromatic acids and in addition a "salicyl-esterase". Most livers split TB at a very high rate, known for a long time in enzyme chemistry.

d) *Kidney*

The kidney experiments have also given results differing from those obtained with other tissues. The ChE contents are low in cow and guinea pig and in both cases BzCh is split at a higher rate than ACh and MeCh. It was found in the studies on the blood of *Spirographis* (p. 62) that the esters of salicylic acid (ASaCh and ASa) were hydrolysed at a much higher rate than any other ester investigated. As regards kidney extracts, similar results were obtained. The guinea-pig kidney is the only material used in this investigation that hydrolyses ASa at a higher rate than other esters. It seems justifiable to assume the presence of a "salicyl-esterase", in the same way as in the case of the polychaete blood. ASaCh is hydrolysed also at a high rate, and proofs have been offered that only the acetyl-salicylic linkage is split. Allowing the reaction to go to completeness, it was stopped when 301 μ l. CO₂ had been evolved in an ASaCh(3) solution. This is almost exactly equal to the calculated value (296.5 μ l.) for a complete destruction of one of the ester linkages in this substrate (see Fig. 30 on p. 143). This linkage, most probably, is the acetyl-salicylic one, for physostigmine does not inhibit the hydrolysis of ASaCh, and BzCh is split at a relatively low rate.

Furthermore, the cow kidney is believed to contain a "salicyl-esterase", the concentration of which is lower than in the kidney of guinea pig. Moreover, cow kidney splits TB at a very high rate.

e) *Intestine*

An enzyme occurs in high concentration in the small intestine of guinea pig hydrolysing TB. On the whole, the esterase activ-

ities of this material resemble in many ways those of kidney. ASaCh is split at a high rate. The activities towards MeCh, BzCh, ASa, and AAn are of about the same range, 15 per cent of the activity towards TB; the activity towards ACh is somewhat higher. It is supposed that a specific ChE is present in the intestine and in addition an unspecified esterase.

f) *Air Bladder of Cod*

The ChE activity of the air bladder is of interest in connection with the problem of the nervous regulation of the bladder. It has been known for a long time that an intact vagus innervation is necessary for the maintenance of a normal function of the bladder. Hence ACh might be a main factor of this function. The ChE activity of the air bladder, demonstrated here for the first time, provides further support for this supposition. The activity, which is rather low, was measured in the tunica interna of the bladder and in the gas gland.¹ The b_{30} values, calculated for 100 mg. tissue, were 35 and 22 respectively, in using ACh as substrate, and 20 and 19, in using MeCh. Unfortunately the activity towards BzCh was not studied (insufficient amount of material at disposal), but the relatively high rate of the hydrolysis of MeCh proposes the enzyme to be a specific ChE. In such a case, ACh as a main factor of the function of the air bladder is further supported, in accordance with the theory of NACHMANSOHN (1945).

2. *Tissues of Invertebrates*

a) *Helix pomatia*

The ChE activities of various tissues of the edible snail have been reported previously (AUGUSTINSSON, 1946 c). In the hydrolysis of ACh the *dart sac* was found surprisingly active. This tissue has the highest concentration of ChE found in any tissue studied in this investigation (Table 16). The activity expressed in Q units (p. 24) is about 15; in comparison, Q for the frog muscle is 0.4—0.6, for the sympathetic ganglia of dogs 11—19, for the electric organs of the common ray and the electric eel 3—10 and 90—150 respectively. As far as the author is aware, very little is known about the structure and functions of the dart sac. Histological investigations have shown that this organ is composed

¹ This material was prepared by Dr. RAGNAR FÄNGE, Department of Zoology, University of Lund.

of smooth muscles richly supplied with nerve fibres (BODIAN staining method).¹ No autonomic drugs (ACh, MeCh, CbCh, adrenaline, histamine), even in high concentrations, caused the organ to contract.² Nor did ACh cause any action when the dart sac was pretreated with physostigmine.

The ChE activity of the dart sac is quite different from that of the *Helix* blood. MeCh, BzCh, and AAn are split, at low rates relatively to ACh; MeCh is destroyed at a higher rate than BzCh, but nevertheless the enzyme has not the characteristics of the specific ChE (p. 122). ASaCh is hydrolysed very rapidly. The sac contains, in all likelihood, an esterase which splits TB, but not EA, and which is not engaged in the hydrolysis of ACh.

In extracts containing 1 g. of the dart sac in 20 ml. R₃₀, the ChE was precipitated by ammonium sulphate at 50 per cent saturation. The yield in this process was 100 per cent. The precipitate, washed with ammonium-sulphate solution, was dissolved in water and the solution used for further experiments after the dialysis against distilled water. The enzyme was adsorbed on infusorial earth, but no satisfactory procedure has yet been found for eluting the enzyme.

b) *Sepia officinalis*

The "liver" or digestive gland of the cuttlefish contains a ChE with a high activity towards ACh; the activities towards MeCh and BzCh are much lower. TB and ASaCh are hydrolysed at high rates, ASa, AAn, and EA at lower ones. It has been shown (p. 151) that this material contains at least two enzymes, one hydrolysing ACh and one TB.

c) *Maja squinado*

The abdominal muscles of the spider crab have very low ChE activity. Also other esters are hydrolysed at low rates.

d) *Balanoglossus clavigerus*

The ChE activities of different parts of this rare Enteropneusta are quite low. In the proboscis and the collar region, the enzyme seems to be of the specific type, hydrolysing ASaCh at about the same rate as ACh. Scarcely any ChE occurs in the outgrowths of

¹ These investigations have been performed in collaboration with Prof. AXEL PALMGREN, Department of Anatomy, Royal Veterinary College, Stockholm.

² The action of drugs on the dart sac was studied in collaboration with Dr. CARL G. SCHMITERLÖW, Department of Physiology and Pharmacology, Royal Veterinary College, Stockholm.

the alimentary canal or the "liver-sacs". These outgrowths, however, hydrolyse ASaCh and EA at a relatively high rate. It may be the acetyl-ester linkage in ASaCh that is broken and the same enzyme responsible for the hydrolysis of ASaCh and EA.

e) *Sagartia parasitica*

The ChE activity has been determined in those parts of this anemone which contain a well-developed diffuse nerve net. At very low rate, ACh, BzCh, and MeCh are split. ASaCh and TB, on the other hand, are hydrolysed at high rates, and it may be supposed that it is the same enzyme which catalyses these reactions.

Sagartia belongs to a group of low invertebrates (sea anemones), known to be perhaps the commonest and largest forms with a single nervous system, a complete columnar nerve net. This actinian nerve net resembles physiologically the vertebrate nerve. They differ, however, chiefly from each other in the diffuse conduction of the nerve net and the fact that an excitation wave in the anemone leaves an after-effect that facilitates the transmission of the next excitation wave. It would be an interesting task to examine this extreme "facilitation" in correlation with studies on the ChE activities in the light of the new hypothesis of NACHMANSOHN (1945). Those parts of the anemone body which contain the nerve net, split ACh, and this observation alone should stimulate more comprehensive studies.

f) *Paracentrotus lividus* (Developing Eggs)

Few articles have appeared dealing with the early ontogenetic development of ChE (p. 13). The enzyme activity has been determined by the author in sea-urchin eggs, both fertilised and unfertilised. Preliminary experiments showed that the ChE activity of Echinoplutei larvae is high; unfertilised eggs, on the other hand, were inactive. From a biological point of view, it would be interesting to trace the changes of the ChE concentrations at various stages of development. From a chemical point of view, such experiments would give valuable informations of the problem whether one or more enzymes are present. Therefore, the activities towards different substrates have been measured and compared with each others. Extracts of frozen and dried eggs (p. 45) were used and their esterase activities determined. This procedure is based on the assumption that the esterase(s) is (are) eluted by R_{20} similarly and almost completely, no matter which stage it may be. The total amounts of dissolved protein, however,

TABLE 19. *Esterase Activities of Developing Sea-Urchin Eggs*

| | | Unfertilised
eggs | Hours after fertilisation | | | | | | $\frac{\text{"65"}}{\text{"48"}} \times 100$ |
|--|----------------------|----------------------|---------------------------|------|------|------|------|------|--|
| | | | 7 | 12 | 18 | 30 | 48 | 65 | |
| Per cent nitrogen of the solutions | | 1.00 | 1.26 | 1.09 | 0.86 | 0.92 | 0.66 | 0.46 | |
| ACh | b_{30} | 0 | 8 | 15 | 24 | 103 | 246 | 30 | |
| | $b_{30}/4$ mg. N | 0 | 6.5 | 14 | 28 | 112 | 373 | 65 | 17.4 |
| MeCh | b_{30} | 2 | 7 | 12 | 20 | 89 | 209 | 23 | |
| | % of ACh(b_{30}) | — | 88 | 80 | 83 | 86 | 85 | 77 | |
| | $b_{30}/4$ mg. N | 2 | 5.5 | 11 | 23 | 97 | 317 | 50 | 15.8 |
| ASaCh | b_{30} | 45 | — | 43 | 55 | 133 | 263 | 102 | |
| | $b_{30}/4$ mg. N | 45 | — | 39.5 | 64 | 145 | 399 | 222 | 55.6 |
| ASa | b_{30} | 12 | — | 19 | 19 | — | 50 | 27 | |
| | $b_{30}/4$ mg. N | 12 | — | 17.5 | 22 | — | 76 | 59 | 77.6 |
| TB | b_{30} | 45 | 47 | 65 | 68 | 94 | 168 | 96 | |
| | $b_{30}/4$ mg. N | 45 | 37.5 | 59.5 | 79 | 102 | 255 | 209 | 82.0 |

were not the same for all preparations (see Table 19); for the higher stages (48 and 65 hours) these amounts were about 50 per cent of those of unfertilised eggs and fertilised eggs of early stages. The total nitrogen concentrations of the original freezing-dried preparations were approximately the same. The enzymic activity is expressed as μ l. CO_2 evolved in 30 minutes (b_{30}), this value calculated for 4 mg. nitrogen, which was the amount of N in the experiments with unfertilised eggs. The activities are tabulated in Table 19 and plotted against hours after fertilisation in Fig. 3.

It is biologically interesting that the ChE activity is uniformly absent in unfertilised and fertilised eggs during the first two hours, but at stage 20 hours there is a rise which grows steeper as it progresses to the high values of the stage of "well-developed Plutei larvae with moving intestine" (48 hours). This sudden increase in ChE content may be correlated with the requirement for a rapid cleavage of ACh. For significant correlations have been found to exist between the amount of ChE present and the functional capacity of the ACh-ChE system (p. 13). Now the question arises whether a correlation exists between the development of the nervous system of sea urchins and the formation of ChE, shown, for example, throughout the larval life of *Amblystoma* (SAWYER, 1943) and in developing grasshopper embryos (TAHMISIAN, 1943). This problem will be discussed more fully in due course by T. GUSTAFSON and the present author.

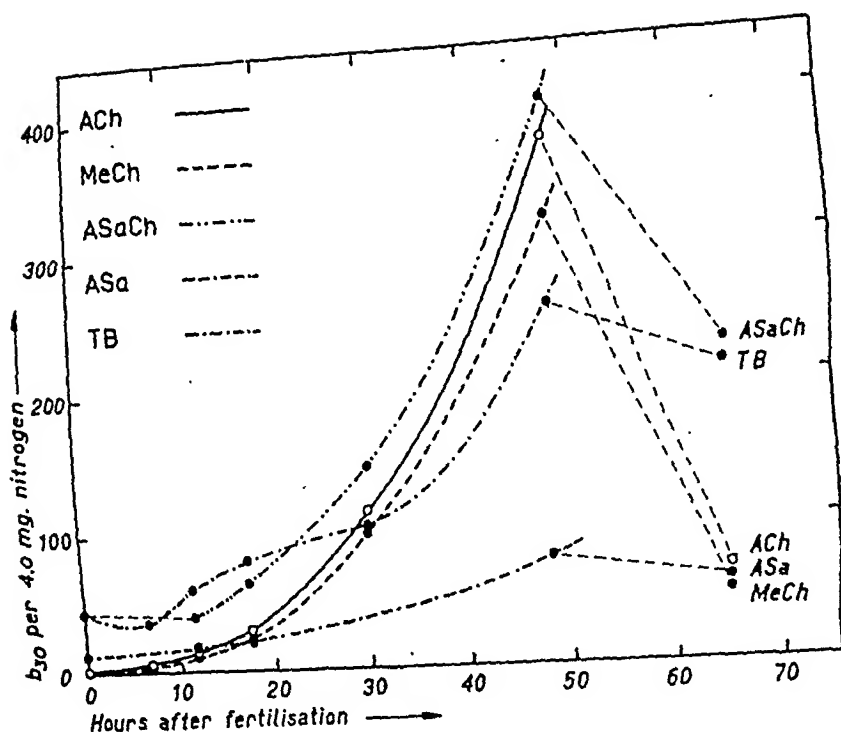


Fig. 3. Esterase activities of developing sea-urchin eggs. Data from Table 19.

As regards the activities towards other choline esters, it is important to note that the hydrolysis of MeCh parallels almost exactly that of ACh. It must therefore be established that the same enzyme of the eggs splits ACh and MeCh. The developing curve for the hydrolysis of TB, on the other hand, is distinct from the corresponding ACh curve. This is a real proof that two distinct enzymes exist in sea-urchin eggs, one splitting choline esters, the other tributyrin. In contradistinction to the ChE, the tributyrinase is present in unfertilised eggs. The concentration of the latter decreases a little during the first two or three hours after fertilisation. Generally, the activity curve is not of such a uniform shape as in the case of ChE. The activity towards ASa varies almost parallel to that towards TB, and it is therefore justifiable to suppose that the same enzyme is responsible for the hydrolysis of the two substrates.

As ever, the hydrolysis of ASaCh is more difficult to analyse. Unfertilised eggs split this substance at about the same rate as they do TB. It is supposed that during the early stages of development, when the ChE concentration is low or zero, the acetyl-ester linkage is split by the tributyrinase. When ChE is formed in high concentrations, it seems as this enzyme plays a great rôle in the

hydrolysis of ASaCh, and then it may be the other ester linkage that is also split.

The 65-hours stage gave surprisingly low values. It may be proposed that the activities are inhibited by some substance of the preparation. Most probably, however, the unexpected low esterase activities are due to an involution, that is, a retrograde change of the embryos at this stage of development. The results are interesting from a chemical point of view. If the values of the 65-hours stage are compared with those of the 48-hours stage, we find the proportions to be of the same range in the hydrolysis of ACh and MeCh (17 and 16 per cent) and in the hydrolysis of TB and ASa (78 and 82 per cent) respectively. The activity of the 65-hours stage towards ASaCh is 56 per cent of the activity of the 48-hours stage, that is, between the values obtained for the choline esters and the ordinary esters. This result offers further proof that two enzymes attack ASaCh and that both ester linkages are split in this double ester.

g) *Marine Animals Belonging to Various Groups of Invertebrates*

The author has previously reported the results obtained with some marine invertebrates (AUGUSTINSSON, 1946 b). In these early investigations, the hydrolysis of ACh was compared with those of MeCh and BzCh in order to offer proofs for the hypothesis of MENDEL and his co-workers (p. 20). These experiments showed, however, that the method suggesting the use of MeCh and BzCh in order to estimate the specificity of ChE is insufficient. Thus, some species hardly split any of the two substrates and still others hydrolyse MeCh at a higher rate than ACh, later on also found with other material (p. 21). An explanation of this will be given in Chapter IX. In a previous paper the author stated that "the reactions used to distinguish between a 'true' and a 'pseudo' ChE with the help of two choline esters do not tell the complete story about the types of this enzyme" (AUGUSTINSSON, 1946 a). The problem seems to be far more complicated (see Chapter XIII).

h) *Bee Venom*

Bee venom had not been incorporated previously in the great collection of materials used in ChE studies when the present author started his investigations. It would be interesting, however, to compare this venom in its relationship to the system ACh-ChE, remembering the marked ChE activity of snake venom (p. 11).

In the few preliminary experiments performed, a 0.1 per cent R_{30} solution of a crude preparation of the venom has been used.

TABLE 20. *Action of Bee Venom on the Enzymic Hydrolysis of ACh*

| Venom
mg. | Enzyme | Substrate | b_{30} |
|--------------|--------------------------|-----------------|----------|
| 0.2 | R ₃₀ | R ₃₀ | 0 |
| " | " | ACh | 1 |
| 2.0 | " | " | 0 |
| — | Purified horse serum ChE | ACh | 179. |
| 0.2 | " " " " | " | 163 |
| — | Purified horse serum ChE | ACh | 201 |
| 2.0 | " " " " | " | 106 |
| — | Cow erythrocytes | ACh | 133 |
| 2.0 | " " | " | 133 |

The results, summarised in Table 20, show that the venom has no ChE activity, determined with ACh as substrate. The venom inhibited the enzyme activity of a purified ChE preparation from horse serum, but it seems to have no effect on the enzyme of red blood cells. Other experiments have not been performed with this material, as it is the supposition of the author that bee venom is not worthy of particular interest in ChE studies.

Only when the experimental work was finished was the author informed of the results of ZELLER, KOCHER and MARITZ (1944) who reported no ChE activity for the bee venom. In addition these authors observed that bee venom and scorpion venom as well inhibit the serum ChE. The short communication, however, does not contain any numerical data.

CHAPTER VII

THE COURSE OF ENZYMIC HYDROLYSIS OF CHOLINE ESTERS BY ESTERASES OF VARIOUS SOURCES

A. INTRODUCTION

It has been stated that both the non-enzymic and enzymic hydrolysis of ACh revert to the kinetics of a first-order reaction. This has been pointed out, for instance, by VAHLQUIST (1935), ABDON and UVNÄS (1937), CLARK *et al.* (1938), and RIECHERT and SCHNARRENBERGER (1942). Some authors, however, found higher values of the reaction constant in the later periods of the enzymic hydrolysis (PLATTNER *et al.*, 1928; PLATTNER & HINTNER, 1930; ZIFF, JAHN & RENSHAW, 1938; CLARK & RAVENTÓS, 1938). A comprehensive study on the kinetics of the system ACh-ChE has been performed by

STRAUS and GOLDSTEIN (1943) and GOLDSTEIN (1944). They stated that the enzymic hydrolysis of ACh as a first-order reaction is actually true only under certain definite limited conditions. In all these previous investigations, blood serum was used as enzyme preparation. The reaction kinetics of the enzymic hydrolysis of choline esters have not been studied previously with ChE from other sources.¹

The discovery that ChE is not to be regarded as a single entity, entitles one to re-examine the kinetics of the enzymic hydrolysis of ACh and other choline esters by various ChE preparations. It was shown in preliminary experiments, the results of which have been recorded in the foregoing Chapter VI, that the choline-ester splitting enzymes from various sources present considerable differences as regards their activities towards ACh and other choline esters. It is to be expected that these facts might be reflected in the course of reactions. Moreover, it has been found previously that one type of enzyme, the specific ChE, is inhibited by excess of substrate; the equilibrium between enzyme and substrate according to the law of mass action is disturbed. The inactivation of the enzyme by temperature and choline, produced during the hydrolysis, may also give rise to deviations from the characteristics of the laws of zero- and first-order reactions. Therefore, it is to be expected that the majority of ChE reactions are not simply zero- or first-order reactions. In the great series of experiments carried out, it was found, however, that many reactions presented typical properties as regards their kinetics. It is the purpose of the following discussion to show that such characteristics really exist and that the reactions may be classified according to these characteristics. The experiments were performed from a comparative point of view and the results used in the drawing of conclusions as to the type of choline-ester splitting enzymes.

The course of reactions were studied in the hydrolysis of choline esters and, in some cases, of tributyrin catalysed by esterases of various tissues. In comparison, the non-enzymic hydrolysis of some substrates were investigated. In each case the reaction constants of proposed zero- and first-order reactions were calculated according to

$$k_0 = \frac{x}{t} \text{ and } k_1 = \frac{1}{t} \log \frac{a}{a-x} \quad (1 \text{ a, b})$$

¹ SCHAEFER (1947), in a recent paper, has given interpretation on the kinetics of the ACh hydrolysis by blood serum and erythrocytes.

² For the sake of convenience, the first-order reaction constant is expressed in this way, though not strictly correct; the true constant is $k_1 \times 2.303$.

respectively. k_0 and k_1 are the reaction constants. The initial concentration of the substrate, a , and the amount of substrate hydrolysed, x , after the time t (minutes) are expressed in $\mu\text{l. CO}_2$. Thus a is the amount of CO_2 which is theoretically evolved if the reaction is allowed to go to completeness. The a values of all substrate solutions used are listed in Table 9 and found also in some cases in the Tables of this Chapter.

It was observed that the reaction constants, both k_0 and k_1 , were not really "constants" in most cases. For this reason, the activity of ChE has been determined at a relatively high substrate concentration and at low enzyme concentration and for a short time and the initial slope of the activity-time curve, minus the slope of the corresponding curve for non-enzymic hydrolysis, taken as an expression of the enzyme activity. The extrapolated 30 minute value was taken as unit, symbolised by b_{30} . These b_{30} values are also shown in the following Tables. The advantage of this arrangement is that the amount of substrate hydrolysed is limited in such a way that the substrate concentration remains high and so the enzyme acts at maximum activity. Moreover, the short reaction period prevents the accumulation of choline which may be supposed to inhibit the enzyme competitively.

B. KINETICS OF NON-ENZYMIC HYDROLYSIS

Confirming previous results of other investigators the non-enzymic hydrolysis of ACh was found to revert to the kinetics of a first-order reaction. k_1 of the hydrolysis in the six ACh solutions used are found in Table 23. During the first 30 minute period the relatively low rate of this reaction was proportional to time. This was also true for the other substrates used. The rates of non-enzymic hydrolysis were expressed in $\mu\text{l. CO}_2$ per 30 minutes and these values are listed in Table 9.

It was reported in Chapter V that both ester linkages in ASaCh are split non-enzymically. More detailed data of the spontaneous hydrolysis of an ASaCh(3) solution are found in Table 21. In the beginning of this reaction its rate was directly proportional to time ($k_0 \approx 0.25$). The first-order reaction constant was calculated, presuming that both ester linkages were split, and found to fall off slowly during the reaction up to 50 per cent hydrolysis. The constant was also calculated for the breakdown of only one of the

TABLE 21. *The Course of Non-Enzymic Hydrolysis of ASaCh*
 $a = 296.5 \mu\text{l.}$

| Time
(min.)
t | $\mu\text{l. CO}_2$
x | Hydrolysis % | | $k_0 \times 10^3$ | $k_1 \times 10^4$ | |
|-----------------------|----------------------------|--------------|------|-------------------|-------------------|------|
| | | (a) | (2a) | | (a) | (2a) |
| 20 | 5 | 1.69 | 0.85 | 250 | 3.70 | 1.85 |
| 40 | 10 | 3.38 | 1.69 | 250 | 3.70 | 1.85 |
| 60 | 14 | 4.62 | 2.36 | 233 | 3.42 | 1.73 |
| 142 | 32 | 10.80 | 5.40 | 225 | 3.50 | 1.70 |
| 217 | 45.5 | 15.36 | 7.68 | 210 | 3.50 | 1.60 |
| 531 | 98.5 | 33.2 | 16.6 | 186 | 3.30 | 1.48 |
| 983 | 156 | 53.6 | 26.3 | 159 | 3.39 | 1.35 |
| 1 679 | 244 | 82.4 | 41.2 | 145 | 4.50 | 1.37 |
| 2 703 | 324 | — | 54.6 | 120 | — | 1.27 |
| 2 940 | 343 | — | 57.8 | 117 | — | 1.27 |

linkages and found to be fairly "constant" during the main part of reaction.

C. STUDIES AT VARIOUS ENZYME CONCENTRATIONS

The enzymes have been used in low concentrations in order to obtain a straight-line relationship between reaction rate and time. In many cases such a relationship holds for a wide range of enzyme concentrations, provided that the substrate concentration is not too low (*e.g.*, ACh-blood serum). In still other cases, a straight line has not been obtained even at very low enzyme concentrations and high substrate concentrations (*e.g.*, TB-guinea-pig liver). When the reaction rate fell off quickly already from the beginning of the reaction, the initial slope of the activity-time curve could not be evaluated adequately. In such cases the activity was expressed as b_{30} , obtained by reading the 30 minute value on the bent curve and subtracting the corresponding value of the non-enzymic hydrolysis. Such values are placed in parentheses in Tables 10 and 16. This procedure, however, was not needed very often.

Two examples of the hydrolysis at various enzyme concentrations are found in Table 22 which lists the values of k_0 and b_{30} for 40 minute periods, immediately following the addition of enzyme to substrate. k_0 was calculated for the total (non-enzymic + enzymic) hydrolysis, b_{30} for the enzymic hydrolysis. Regarding the ACh hydrolysis by horse blood cells, a straight line relationship was obtained at all enzyme concentrations (k_0 constant). This is characteristic of enzymes with high affinity and holds

TABLE 22. *The Course of Hydrolysis at Various Enzyme Concentrations*
Cf. Figs. 4 and 5.

| Substrate | | ACh(3) | | | | ASaCh(3) | | |
|-----------------------|-------------------------|----------------------|------|-------------------|----------|----------------------------|-------------------|----------|
| Enzyme | | Erythrocytes (horse) | | | | Liver (guinea pig) | | |
| Relative enzyme conc. | Time (min.)
<i>t</i> | $\mu\text{l. CO}_2$ | | $k_0 \times 10^2$ | b_{30} | $\mu\text{l. CO}_2$
x | $k_0 \times 10^2$ | b_{30} |
| | | Actual release | x | | | | | |
| 1 | 7 | 42.5 | 23.5 | 336 | 93 | 43.5 | 622 | 180 |
| | 14 | 64 | 45 | 322 | | 87 | 622 | |
| | 23 | 97 | 78 | 339 | | 135 | 587 | |
| | 30 | 119 | 100 | 333 | | 166 | 553 | |
| | 40 | 152 | 133 | 333 | | 202 | 505 | |
| $1/2$ | 7 | 24 | 15 | 214 | 57 | 26 | 371 | 106 |
| | 14 | 38.5 | 29.5 | 211 | | 52 | 371 | |
| | 23 | 57.5 | 48.5 | 211 | | 84 | 365 | |
| | 30 | 72.5 | 63.5 | 212 | | 102 | 340 | |
| | 40 | 95 | 86 | 215 | | 130.5 | 326 | |
| $1/4$ | 7 | 13.5 | 8.5 | 121.5 | 29 | 14 | 200 | 53 |
| | 14 | 22 | 17 | 121.5 | | 28.5 | 204 | |
| | 23 | 33 | 28 | 121.5 | | 46 | 200 | |
| | 30 | 42 | 37 | 123.5 | | 59 | 197 | |
| | 40 | 54 | 49 | 122.5 | | 76 | 190 | |
| $1/8$ | 7 | 8 | 5 | 71.4 | 15 | 9 | 129 | 26 |
| | 14 | 13.5 | 10.5 | 75.0 | | 16.5 | 118 | |
| | 23 | 20 | 17 | 74.0 | | 25.5 | 111 | |
| | 30 | 25 | 22 | 73.3 | | 32.5 | 108 | |
| | 40 | 33 | 30 | 75.0 | | 42.5 | 106 | |

in most cases when the enzymic hydrolysis is depressed by excess of substrate.

Regarding erythrocyte haemolysate as an esterase preparation (Table 22), a few words must be said about the spontaneous hydrolysis which sets in immediately after adding the haemolysate to the bicarbonate solution. This liberation of CO_2 is due to the action of carbonic anhydrase which occurs especially in the erythrocytes. This enzyme is also present in traces in other animal tissues (vertebrate as well as invertebrate) and a small "spontaneous" hydrolysis was therefore also observed in such cases. The action of carbonic anhydrase has ended after about 2—3 minutes. Fig. 4 shows the total hydrolysis of ACh by horse erythrocytes at various enzyme concentrations (cf. Table 22). The curves are typical for the reactions where blood cell haemolysate is used. In such cases the "ChE curves" were extrapolated to zero time and the intercepts subtracted from the 30 minute

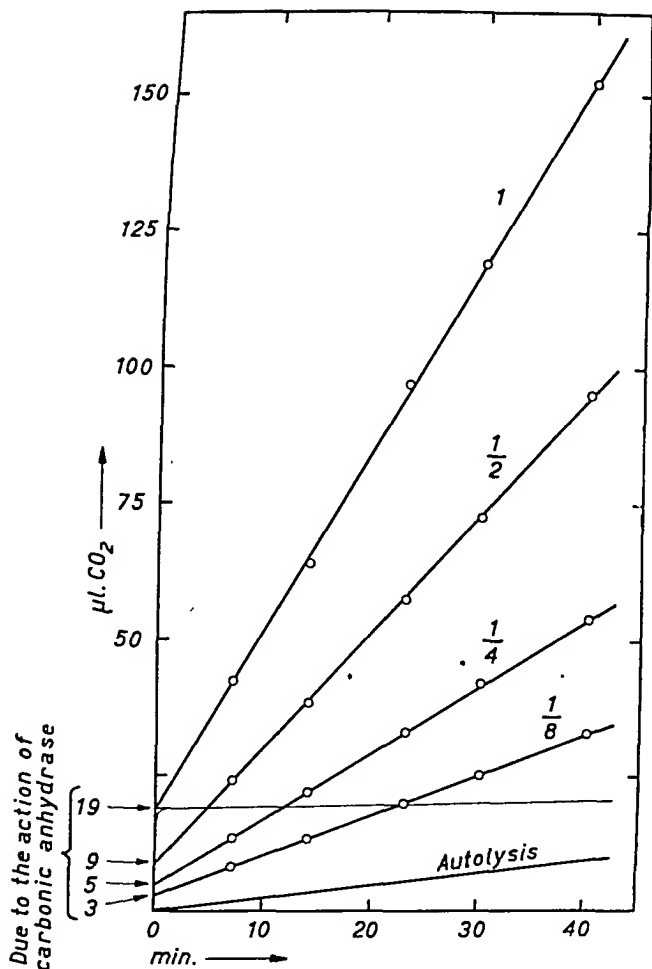


Fig. 4. Total (non-enzymic + enzymic) hydrolysis of ACh by various concentrations of horse erythrocyte ChE. Data from Table 22. Thin line refers to an experiment carried out in the absence of ACh. "Autolysis" means self-decomposition (non-enzymic hydrolysis) of the substrate.

values. The b_{30} values were then calculated in the usual manner by subtracting the amount of CO₂ in μl. evolved during 30 minutes by non-enzymic hydrolysis of the substrate. In Fig. 4 a reaction curve (thin line) for the action of carbonic anhydrase is also shown, obtained by measuring the liberation of CO₂ when the haemolysate was mixed with the bicarbonate solution without any ester. The mixing of enzyme preparation and bicarbonate solution was made as quickly as possible in order to minimize the disturbance of temperature equilibrium. Manometric readings were made at 30 and 60 seconds and afterwards at 1 minute intervals. In the experiment demonstrated in Fig. 4, the amount of CO₂ evolved after 2—3 minutes by the action of carbonic

anhydrase was 19 μ l., that is, the same value as was obtained by extrapolating the corresponding ChE curve to zero time. At the 40 minute period the liberated CO_2 had increased to about 21 μ l.; that increase was too small to be taken notice of in calculating the ChE activity. In the other experiments with various haemolysate concentrations at constant ACh concentration, the extrapolated zero values showed direct proportionality between the amount of CO_2 , liberated by the action of carbonic anhydrase, and enzyme concentration.

In the hydrolysis of ASaCh by guinea-pig liver (Table 22) the reaction rate fell off during the first 40 minute period. This falling off was especially obvious when the initial rate was high, but also at low enzyme concentrations the rate decreased, in opposition to the hydrolysis of ACh by erythrocytes. The k_1 values have not been calculated for the ASaCh hydrolysis as it was not known whether one or both ester linkages were split and thus the initial substrate concentration expressed in a was unknown (cf. p. 77).

The relation between enzyme activity and enzyme concentration will be discussed in Chapter VIII.

D. STUDIES AT VARIOUS SUBSTRATE CONCENTRATIONS

The reaction course has been studied for the enzymic hydrolysis of ACh by horse serum ChE, cow erythrocyte ChE, dog brain, the blood and the dart sac of *Helix pomatia*, and of BzCh by guinea-pig liver. These six examples have been selected because they gave the most characteristic patterns throughout this investigation. The results are recorded in Tables 23—28. They show the total hydrolysis of the substrate at various substrate concentrations. It may be noticed that the constants were calculated for the total hydrolysis, that is, for the enzymic and non-enzymic hydrolysis taken together. Therefore, k_0 and k_1 are the sums of two constants respectively. In comparison with the rate of enzymic hydrolysis, the rate of non-enzymic hydrolysis was low and reverted to the law of a first-order reaction (k_1 constant). The rate of non-enzymic hydrolysis was really so low that the amount of CO_2 spontaneously evolved was practically to be regarded as proportional to time (k_0 constant). Because of this and as the reactions were studied only during a short period (40 minutes), the constants calculated for the total hydrolysis

TABLE 23. *The Course of Hydrolysis of ACh by Horse Serum ChE (purified) at Various Substrate Concentrations**k*₁ values of enzymic hydrolysis in italics.

Solution No. 1 2 3 4 5 6
*k*₁ × 10⁴ of non-enzymic hydrolysis
 (mean values)..... 0.9 1.4 2.1 5.0 12.1 30.9

| Total hydr. corresponds to a μl. | Time (min.)
<i>t</i> | μl. CO ₂
<i>x</i> | Hydro-lysis % | <i>k</i> ₀ × 10 ² | <i>k</i> ₁ × 10 ⁴ | | <i>b</i> _∞ |
|----------------------------------|-------------------------|---------------------------------|---------------|---|---|-------------|-----------------------|
| ACh(1)
<i>a</i> = 4 928 | 7 | 58 | 1.18 | 828 | 7.29 | <i>6.39</i> | 204 |
| | 14 | 114 | 2.31 | 814 | 7.29 | | |
| | 23 | 187 | 3.80 | 813 | 7.30 | | |
| | 30 | 243 | 4.93 | 810 | 7.33 | | |
| | 40 | 320 | 6.50 | 800 | 7.30 | | |
| ACh(2)
<i>a</i> = 1 479 | 7 | 50 | 3.38 | 714 | 21.3 | <i>19.9</i> | 186 |
| | 14 | 100 | 6.76 | 714 | 21.7 | | |
| | 23 | 163 | 11.0 | 708 | 22.0 | | |
| | 30 | 205 | 13.9 | 683 | 21.7 | | |
| | 40 | 279 | 18.9 | 698 | 22.8 | | |
| ACh(3)
<i>a</i> = 493 | 7 | 41 | 8.33 | 586 | 54.0 | <i>51.9</i> | 153 |
| | 14 | 82 | 16.7 | 586 | 56.7 | | |
| | 23 | 130 | 26.4 | 565 | 57.9 | | |
| | 30 | 160 | 32.5 | 533 | 56.9 | | |
| | 40 | 203 | 41.2 | 508 | 57.7 | | |
| ACh(4)
<i>a</i> = 148 | 7 | 35 | 23.6 | 500 | 167 | <i>162</i> | 118 |
| | 14 | 66.5 | 44.9 | 475 | 185 | | |
| | 23 | 92 | 62.2 | 400 | 184 | | |
| | 30 | 109 | 73.6 | 364 | 193 | | |
| | 40 | 123 | 83.1 | 308 | 193 | | |
| ACh(5)
<i>a</i> = 49.3 | 7 | 19 | 38.5 | 272 | 302 | <i>290</i> | 53 |
| | 14 | 33 | 66.9 | 236 | 344 | | |
| | 23 | 44 | 89.3 | 191 | 422 | | |
| | 30 | 48 | 97.4 | 160 | 528 | | |
| | 40 | 49 | 99.5 | 123 | 555 | | |
| ACh(6)
<i>a</i> = 14.8 | 7 | 9 | 60.8 | 129 | 581 | <i>551</i> | 11 |
| | 14 | 12.5 | 84.5 | 89.3 | 578 | | |
| | 23 | 14 | 94.6 | 60.8 | 551 | | |
| | 30 | 15 | 100 | — | — | | |

may give clear pictures of the types of the enzymic reactions. Moreover, the purpose of this series of experiments has been to compare ChE reactions, the kinetics of which are characteristic.

Blood serum. It was shown in preliminary experiments that the reaction velocity falls off with time of hydrolysis when blood serum catalyses the hydrolysis of ACh. Table 23 lists the results from such an experiment carried out at various initial concentrations of the substrate and at the same concentration of a purified

ChE preparation from horse serum. In each of the six experiments the values of k_0 were lowered with the time of reaction. This was more pronounced at lower initial substrate concentrations. When these concentrations were high, k_0 was changed only a little and the amount of hydrolysed ester almost directly proportional to time. The lowering of k_0 at lower ACh concentrations, however, was not very great, and the b_{30} values of the enzymic hydrolysis were easily obtained by extrapolation. The b_{30} (and k_0) values successively decreased from high to low ACh concentrations, said to be characteristic of the non-specific ChE. More details about the ChE activity at various substrate concentrations will be discussed in Chapter IX.

The values of the first-order reaction constant, k_1 , were higher in the later periods of the hydrolysis than in the beginning. This rise was more marked at low initial ACh concentrations. At high concentrations and when the percentage hydrolysis was low, k_1 was practically constant. The values of k_1 (enzymic) in the beginning of the hydrolysis for each ACh concentration range increased about 85 times when the substrate concentration decreased from 10^{-1} to 3×10^{-4} M.

It is assumed throughout this investigation that the action of ChE obeys the MICHAELIS-MENTEN theory. If, at constant pH, the substrate concentration is high enough during the main part of the reaction to saturate the enzyme, k_0 would remain constant. It is reasonable to suppose that this would be the case, at least at high substrate concentrations and no inhibition by the products of reaction, for such a highly active enzyme as ChE. If the substrate concentration is low and hence the ChE not saturated completely with ACh, the hydrolysis might be a first-order reaction. Neither of these cases applies to the hydrolysis of ACh by blood serum, and therefore the initial reaction velocity, expressed as b_{30} , is the most adequate way to state the enzyme activity. A lowering of k_0 , even at rather high substrate concentrations, is most probably due to an inhibition of the enzyme by the products of reaction. It may be assumed that choline, one of the two products, unites reversibly with ChE and because of that the velocity falls off during runs. The effect of choline of various concentrations has been investigated for a series of ACh-ChE systems; the results of such experiments will be discussed in Chapter X. Acetic acid does not inhibit the ChE activity. The

TABLE 24. *The Course of Hydrolysis of ACh by Cow Erythrocyte ChE (purified) at Various Substrate Concentrations*

Cf. Table 23.

| Total hydr.
corresponds
to a μ l. | Time
(min.)
t | μ l. CO ₂
x | Hydro-
lysis
% | $k_0 \times 10^3$ | $k_1 \times 10^4$ | b_{30} |
|---|-----------------------|---------------------------------|----------------------|-------------------|-------------------|----------|
| ACh(1)
$a = 4\ 928$ | 7 | 16 | 0.33 | 229 | 2.00 | 38 |
| | 14 | 33 | 0.67 | 236 | 2.14 | |
| | 21 | 49 | 0.99 | 233 | 2.09 | |
| | 30 | 70 | 1.42 | 233 | 2.07 | |
| | 40 | 94 | 1.91 | 235 | 2.10 | |
| ACh(2)
$a = 1\ 479$ | 7 | 19 | 1.28 | 272 | 8.00 | 69 |
| | 14 | 39 | 2.64 | 279 | 8.28 | |
| | 21 | 59 | 3.99 | 281 | 8.43 | |
| | 30 | 83 | 5.61 | 277 | 8.37 | |
| | 40 | 110 | 7.44 | 275 | 8.40 | |
| ACh(3)
$a = 493$ | 7 | 23 | 4.67 | 329 | 29.7 | 96 |
| | 14 | 48 | 9.74 | 343 | 31.8 | |
| | 21 | 72 | 14.6 | 343 | 32.6 | |
| | 30 | 103 | 20.9 | 344 | 34.0 | |
| | 40 | 136 | 27.6 | 340 | 35.1 | |
| ACh(4)
$a = 148$ | 7 | 34 | 23.0 | 486 | 162 | 145 |
| | 14 | 68 | 45.9 | 486 | 191 | |
| | 21 | 95 | 64.2 | 453 | 212 | |
| | 30 | 122.5 | 82.8 | 408 | 255 | |
| | 40 | 136 | 91.9 | 340 | 273 | |
| ACh(5)
$a = 49.3$ | 2.5 | 12 | 24.4 | 480 | 486 | 140 |
| | 4.5 | 22 | 44.6 | 489 | 570 | |
| | 7 | 34 | 69.0 | 486 | 727 | |
| | 14 | 50 | 100 | — | — | |
| ACh(6)
$a = 14.8$ | 2.5 | 7 | 47.3 | 280 | 1 110 | 82 |
| | 4.5 | 12.5 | 84.5 | 278 | 1 800 | |
| | 7 | 14 | 94.6 | 200 | 1 810 | |
| | 14 | 15 | 100 | — | — | |

serum ChE is not inactivated by heat at 37.5°C in 3—4 hours and pH does not change during the reaction.

Erythrocytes. In the hydrolysis of ACh by blood-cell ChE the results were not of the same kind as those obtained with the serum enzyme (Table 24). At almost all ACh concentrations, the velocities were not at all, or very little changed during the 40 minute period, which means that all activity-time curves were very nearly straight lines and that the values of k_0 remained fairly constant even at low ACh concentrations and near the end of the hydrolysis. Most characteristic of this ChE is the lower reaction velocities at high initial substrate concentrations than

TABLE 26. *The Course of Hydrolysis of ACh by Helix Blood (40 μ l.) at Various Substrate Concentrations*

Cf. Table 23.

| Total hydr. corresponds to a μ l. | Time (min.)
t | μ l. CO ₂
x | Hydro-lysis % | $k_0 \times 10^2$ | $k_1 \times 10^1$ | | b_{30} |
|---|--------------------|---------------------------------|---------------|-------------------|-------------------|-------|----------|
| ACh(1)
$a = 4\ 928$ | 6 | 45 | 0.91 | 75.0 | 6.67 | 5.77 | 194 |
| | 13 | 98 | 1.99 | 75.4 | 6.77 | | |
| | 20 | 152.5 | 3.10 | 76.2 | 6.85 | | |
| | 30 | 226 | 4.58 | 75.3 | 6.80 | | |
| | 38 | 286 | 5.80 | 75.2 | 6.82 | | |
| | 47 | 350 | 7.10 | 74.5 | 6.81 | | |
| ACh(2)
$a = 1\ 479$ | 6 | 62.5 | 4.22 | 104 | 31.2 | 29.8 | 324 |
| | 13 | 132.5 | 8.96 | 102 | 31.4 | | |
| | 20 | 198 | 13.4 | 99.0 | 31.3 | | |
| | 30 | 277 | 18.7 | 92.3 | 30.0 | | |
| | 38 | 348 | 23.5 | 91.5 | 30.6 | | |
| | 47 | 425 | 28.7 | 90.4 | 31.2 | | |
| ACh(3)
$a = 493$ | 6 | 82.5 | 16.7 | 138 | 132 | 130 | 413 |
| | 13 | 172 | 34.9 | 132 | 143 | | |
| | 20 | 248 | 50.3 | 124 | 152 | | |
| | 30 | 363 | 73.6 | 121 | 193 | | |
| | 38 | 415 | 84.2 | 109 | 211 | | |
| | 47 | 452 | 91.6 | 96.2 | 229 | | |
| ACh(4)
$a = 148$ | 2.5 | 36 | 24.3 | 144 | 483 | 478 | 419 |
| | 6 | 85 | 57.5 | 142 | 619 | | |
| | 13 | 142 | 95.9 | 109 | 1 065 | | |
| | 20 | 145.5 | 98.3 | 72.8 | 885 | | |
| | 30 | 147 | 99.3 | — | — | | |
| | 38 | 147 | 99.3 | — | — | | |
| ACh(5)
$a = 49.3$ | 2.5 | 30 | 60.8 | 120 | 1 630 | 1 618 | 356 |
| | 6 | 48 | 97.4 | 80 | 2 640 | | |
| | 13 | 50 | 100 | — | — | | |
| ACh(6)
$a = 14.8$ | 2.5 | 8 | 54.1 | 32 | 1 353 | 1 323 | 120 |
| | 6 | 15 | 100 | — | — | | |

time curves were straight lines except at low ACh concentrations and when the hydrolysis had reached more than 80 per cent. As with the activity of the erythrocyte ChE, that of the brain enzyme was depressed by high ACh concentrations. k_1 (enzymic) increased with time of reaction and was 1 400 times higher at the lowest initial ACh concentration than at the highest one.

Helix blood. The velocities of the hydrolysis of ACh catalysed by *Helix* blood were fairly constant during runs of reactions (Table 26) up to nearly complete destruction of the substrate (cf. Fig. 37, p. 153). The enzyme of this invertebrate blood, as well as

TABLE 28. *The Course of Hydrolysis of BzCh by Guinea-Pig Liver (100 mg.) at Various Substrate Concentrations*

Cf. Table 23.

| Total hydr.
corresponds
to a μ l. | Time
(min.)
t | μ l. CO ₂
x | Hydro-
lysis
% | $k_0 \times 10$ | $k_1 \times 10^4$ | b_{30} |
|---|-----------------------|---------------------------------|----------------------|-----------------|-------------------|----------|
| BzCh(1)
$a = 3\ 683$ | 6 | 247 | 6.72 | 412 | 50.3 | 1 372 |
| | 12 | 411 | 11.2 | 342 | 43.0 | |
| | 20 | 559 | 15.2 | 280 | 35.8 | |
| | 30 | 678 | 18.4 | 226 | 29.4 | |
| | 40 | 760 | 20.6 | 190 | 25.1 | |
| BzCh(2)
$a = 1\ 103$ | 6 | 117 | 10.6 | 195 | 81.2 | 608 |
| | 12 | 216 | 19.6 | 180 | 78.9 | |
| | 20 | 306 | 27.7 | 153 | 70.4 | |
| | 30 | 388 | 35.2 | 129 | 62.8 | |
| | 40 | 442 | 40.0 | 111 | 55.4 | |
| BzCh(3)
$a = 368.5$ | 6 | 49.5 | 13.4 | 82.5 | 104 | 292 |
| | 12 | 93 | 25.3 | 77.5 | 106 | |
| | 20 | 121.5 | 33.0 | 60.8 | 86.9 | |
| | 30 | 160 | 43.5 | 53.3 | 82.7 | |
| | 40 | 189 | 51.3 | 47.3 | 78.1 | |
| BzCh(4)
$a = 110.5$ | 6 | 18.5 | 16.7 | 30.8 | 132 | 98 |
| | 12 | 35 | 31.7 | 29.2 | 138 | |
| | 20 | 52 | 47.1 | 26.0 | 138 | |
| | 30 | 67 | 60.7 | 22.3 | 135 | |
| | 40 | 78 | 70.6 | 19.5 | 133 | |
| BzCh(5)
$a = 36.8$ | 2.5 | 5.5 | 14.9 | 22.0 | 280 | 54 |
| | 4.5 | 9 | 24.5 | 20.0 | 272 | |
| | 6 | 11.5 | 31.2 | 19.2 | 271 | |
| | 12 | 19 | 51.6 | 15.8 | 262 | |
| | 20 | 26 | 70.7 | 13.0 | 267 | |
| BzCh(6)
$a = 11.0$ | 30 | 31.5 | 85.6 | 10.5 | 281 | 37 |
| | 2.5 | 4 | 36.4 | 16.0 | 786 | |
| | 4.5 | 6 | 54.5 | 13.3 | 760 | |
| | 6 | 8 | 72.8 | 13.3 | 942 | |
| | 12 | 11 | 100 | — | — | |

contradistinction to the *Helix*-blood enzyme, the ChE of the dart sac was not inhibited by excess of substrate. The value of the first-order reaction constant at initial ACh concentration increased about 60 times from the lowest to the highest concentration range.

Guinea-pig liver. The course of the hydrolysis of BzCh by guinea-pig liver differed greatly from the kinetics of the ACh reactions reported above (Table 28). The reaction velocities fell off definitely with time. At low BzCh concentrations this falling off was characteristic of a first-order reaction. At high substrate concentrations

the velocities decreased so rapidly that the first-order reaction constant also fell off. The values of k_0 and b_{30} respectively increased considerably with increasing initial BzCh concentration. The k_1 value in the beginning of the enzymic hydrolysis was 15 times higher at low than at high BzCh concentrations.

A brief discussion of these results will be found in the next section.

E. COMPARISON OF THE HYDROLYSIS OF VARIOUS ESTERS

The reaction kinetics of the hydrolysis of certain esters in addition to ACh by the enzyme preparations considered in the foregoing section have also been studied. The purpose of this series of experiments has been to compare the various reactions with each other, rather than to determine the order of the reaction. Because of the uncertainty whether both or only one of the ester linkages in ASaCh were broken, only the values of k_0 have been calculated in these cases; the same was true for TB all the ester linkages of which are, it is true, identical but *a priori* may not be split at the same rate. The results of these experiments are listed in Table 29.

Blood plasma. The kinetics of the hydrolysis of BzCh, ASaCh, and TB by horse plasma were much the same as those of ACh; the reaction velocities fell off slowly.

Erythrocytes. It was characteristic of the enzymic hydrolysis of MeCh by human erythrocytes that the velocity fell off rapidly with time, in sharp contrast to the corresponding ACh hydrolysis. This falling off was greater than that characteristic of a first-order reaction and consequently k_1 decreased with time. Also the reaction velocity of the ASaCh hydrolysis decreased rapidly. The low rate of the TB hydrolysis was not changed during the 40 minute period.

Brain. The low velocities of the hydrolysis through the action of brain extracts were constant or decreased slowly. At higher enzyme concentrations the falling off was more obvious.

Helix blood. The velocities of the MeCh and ASaCh hydrolysis catalysed by *Helix* blood decreased more rapidly than in the ACh breakdown. In the MeCh reaction this falling off was greater than that characteristic of a first-order reaction, as with the hydrolysis of MeCh by the erythrocyte ChE. k_1 decreased in the MeCh- and increased in the ACh-hydrolysis. Also in the

TABLE 29. *The Course of Hydrolysis of Certain*
Substrate solutions No. 3. Complete hydrolysis

| Enzyme | Horse plasma (100 μ l.) | | | | | | | | | | |
|----------|-----------------------------|------|-----------------|-------------------|----------|------|-----------------|----------|-----------------|----------|-----------------|
| Substr. | ACh | | | | BzCh | | | ASaCh | | TB | |
| <i>t</i> | <i>x</i> | % | $k_0 \times 10$ | $k_1 \times 10^4$ | <i>x</i> | % | $k_0 \times 10$ | <i>x</i> | $k_0 \times 10$ | <i>x</i> | $k_0 \times 10$ |
| 7 | 83 | 16.8 | 119 | 114 | 37 | 10.0 | 52.8 | 70.5 | 101 | 36.5 | 52.1 |
| 14 | 166 | 33.7 | 119 | 128 | 76 | 20.6 | 54.3 | 129 | 92.2 | 75 | 53.6 |
| 21 | 232 | 47.1 | 111 | 132 | 114 | 31.0 | 54.3 | 178 | 84.8 | 112 | 53.3 |
| 30 | 304 | 61.7 | 101 | 139 | 158 | 42.8 | 52.7 | 236 | 78.7 | 149 | 49.7 |
| 40 | 360 | 73.1 | 90 | 143 | 198 | 53.8 | 49.5 | 280 | 70.0 | 190 | 47.5 |
| b_{31} | 353 | | | | 161 | | | 290 | | 160 | |

| Enzyme | Human erythrocytes (200 μ l.) | | | | | | | | | | |
|----------|-----------------------------------|------|-----------------|----------|------|-----------------|-------------------|----------|-----------------|----------|-----------------|
| Substr. | ACh | | | MeCh | | | | ASaCh | | TB | |
| <i>t</i> | <i>x</i> | % | $k_0 \times 10$ | <i>x</i> | % | $k_0 \times 10$ | $k_1 \times 10^4$ | <i>x</i> | $k_0 \times 10$ | <i>x</i> | $k_0 \times 10$ |
| 7 | 75.5 | 15.3 | 108 | 49.5 | 10.5 | 70.7 | 70.8 | 87 | 124 | 10.5 | 15.0 |
| 14 | 161 | 32.6 | 115 | 86 | 18.8 | 61.4 | 64.5 | 139 | 99.4 | 21.5 | 15.4 |
| 21 | 235 | 47.6 | 112 | 116.5 | 25.5 | 55.5 | 60.8 | 171 | 81.5 | 32.5 | 15.5 |
| 30 | 334 | 67.7 | 111 | 145 | 31.7 | 48.3 | 55.2 | 203 | 67.6 | 47 | 15.7 |
| 40 | 448 | 91.0 | 112 | 168 | 36.8 | 42.0 | 49.8 | 224 | 56.0 | 62 | 15.5 |
| b_{30} | 327 | | | (144) | | | | (196) | | 46 | |

| Enzyme | Dog brain (100 mg.) | | | | | | | | | |
|----------|---------------------|------|-------------------|----------|-----|-------------------|----------|-------------------|----------|-------------------|
| Substr. | ACh | | | MeCh | | | ASaCh | | TB | |
| <i>t</i> | <i>x</i> | % | $k_0 \times 10^2$ | <i>x</i> | % | $k_0 \times 10^2$ | <i>x</i> | $k_0 \times 10^2$ | <i>x</i> | $k_0 \times 10^2$ |
| 6 | 14 | 2.8 | 233 | 5.5 | 1.2 | 91.7 | 14 | 233 | 11 | 183 |
| 12 | 27 | 5.5 | 225 | 10.5 | 2.3 | 87.5 | 28 | 233 | 22.5 | 188 |
| 20 | 45.5 | 9.2 | 228 | 17.5 | 3.8 | 87.5 | 47 | 235 | 37 | 185 |
| 30 | 67 | 13.6 | 223 | 26 | 5.7 | 86.6 | 69 | 230 | 56 | 187 |
| 40 | 89 | 18.1 | 223 | 35 | 7.6 | 87.5 | 89.5 | 224 | 74.5 | 186 |
| b_{30} | 60 | | | 25 | | | 63 | | 56 | |

hydrolysis of AAn k_0 fell off, but to such a degree that k_1 was constant.

Dart sac. Similar results were obtained in the reactions of the dart-sac ChE. ACh was split in such a way that k_1 increased and

Esters by Various Enzyme Preparations

corresponds to a $\mu\text{l. CO}_2$ (see Table 9).

| Enzyme | <i>Helix</i> blood (40 $\mu\text{l.}$) | | | | | | | | | | | | |
|----------|---|------|-------------------|----------|------|-------------------|-------------------|----------|-------------------|----------|------|-------------------|-------------------|
| Substr. | ACh | | | MeCh | | | | ASaCh | | AAn | | | |
| <i>t</i> | <i>x</i> | % | $k_0 \times 10^2$ | <i>x</i> | % | $k_0 \times 10^2$ | $k_1 \times 10^4$ | <i>x</i> | $k_0 \times 10^2$ | <i>x</i> | % | $k_0 \times 10^2$ | $k_1 \times 10^4$ |
| 8 | 66 | 13.4 | 825 | 28 | 6.1 | 350 | 34.3 | 15.5 | 194 | 20.5 | 8.7 | 256 | 49.2 |
| 16 | 132 | 26.8 | 825 | 49 | 10.7 | 306 | 30.7 | 28 | 175 | 40 | 16.9 | 250 | 50.2 |
| 24 | 197 | 40.0 | 821 | 66 | 14.4 | 275 | 28.1 | 38 | 158 | 58.5 | 24.8 | 244 | 51.6 |
| 32 | 264 | 53.6 | 825 | 81 | 17.7 | 253 | 26.4 | 45 | 141 | 74 | 31.3 | 231 | 51.0 |
| 40 | 332 | 62.3 | 830 | 89 | 19.6 | 222 | 23.7 | 51 | 128 | 87 | 36.8 | 218 | 49.8 |
| b_{30} | 239 | | | 104 | | | | 45 | | 72 | | | |

| Enzyme | Dart sac (50 mg.) | | | | | | | | | | | |
|----------|-------------------|------|-----------------|-------------------|----------|-----|-----------------|-------------------|----------|-----------------|----------|-----------------|
| Substr. | ACh | | | | MeCh | | | | ASaCh | | TB | |
| <i>t</i> | <i>x</i> | % | $k_0 \times 10$ | $k_1 \times 10^4$ | <i>x</i> | % | $k_0 \times 10$ | $k_1 \times 10^4$ | <i>x</i> | $k_0 \times 10$ | <i>x</i> | $k_0 \times 10$ |
| 8 | 129 | 26.2 | 161 | 165 | 13 | 2.8 | 16.3 | 15.6 | 49.5 | 61.9 | 14.5 | 18.1 |
| 16 | 246 | 49.9 | 154 | 188 | 22.5 | 4.9 | 14.1 | 13.7 | 93.5 | 58.4 | 29.5 | 18.4 |
| 24 | 336 | 68.2 | 140 | 207 | 30 | 6.6 | 12.5 | 12.3 | 131.5 | 54.8 | 43.5 | 18.1 |
| 32 | 386 | 78.4 | 121 | 208 | 36.5 | 8.0 | 11.4 | 11.3 | 166 | 51.9 | 58.5 | 18.3 |
| 40 | 420 | 85.3 | 105 | 208 | 42 | 9.2 | 10.5 | 10.5 | 193 | 48.3 | 73 | 18.3 |
| b_{20} | 453 | | | | 42 | | | | 169 | | 55 | |

| Enzyme | Guinea-pig liver (100 mg.) | | | | | | | | | | | |
|----------|----------------------------|------|-----------------|-------------------|----------|-----------------|----------|------|-----------------|-------------------|----------|-----------------|
| Substr. | BzCh | | | | ASaCh | | ASa | | | | TB | |
| <i>t</i> | <i>x</i> | % | $k_0 \times 10$ | $k_1 \times 10^4$ | <i>x</i> | $k_0 \times 10$ | <i>x</i> | % | $k_0 \times 10$ | $k_1 \times 10^4$ | <i>x</i> | $k_0 \times 10$ |
| 7 | 69 | 18.7 | 98.6 | 128 | 99 | 141 | 68.5 | 13.8 | 97.8 | 92.1 | 226 | 323 |
| 14 | 120 | 32.6 | 86.7 | 122 | 170 | 121 | 128 | 25.7 | 91.5 | 92.2 | 289 | 206 |
| 21 | 153 | 41.6 | 72.9 | 111 | 223 | 106 | 173 | 34.8 | 82.4 | 88.5 | 334 | 159 |
| 30 | 188 | 51.1 | 62.7 | 104 | 273 | 91.0 | 214 | 43.0 | 71.3 | 81.4 | 376 | 125 |
| 40 | 215 | 58.3 | 53.8 | 95.0 | 314 | 78.5 | 234 | 47.0 | 58.5 | 68.9 | 410 | 103 |
| b_{30} | 296 | | | | (266) | | 273 | | | | (376) | |

MeCh in such a way that k_1 fell off. The velocity of the MeCh hydrolysis was slowed down rapidly even at low enzyme concentrations. Also the velocity of the ASaCh reaction fell off rapidly, but the low rate of the breakdown of TB was constant.

Guinea-pig liver. Characteristic of the esterase reactions of the guinea-pig liver was the great falling off of velocities with time. This was true for all four examples, shown in Table 29. In the enzymic hydrolysis of BzCh, as well as those of ASaCh, ASa, and TB, the k_1 values decreased. It is to be noted that the 40 minute value of x ($= 314 \mu\text{l.}$) in the ASaCh hydrolysis is higher than the calculated value for the complete breakdown of one of the ester linkages ($a = 296.5 \mu\text{l.}$). Thus both ester linkages must be attacked by the liver enzyme(s) (see Fig. 30, p. 143).

F. SUMMARY OF CHAPTER VII

We have seen that the kinetics of various ChE reactions vary to a great extent. Nevertheless, there exist certain regularities among the reactions studied. Table 30 shows some general characteristics of a group of ChE-substrate systems. These characteristics will be compared with other properties of the same combinations and discussed in the following Chapters.

A straight line relationship between time and amount of substrate hydrolysed (k_0 constant) may be obtained if the substrate has a high affinity for the enzyme and if neither choline nor temperature has any remarkable effect on the enzyme. This is actually true for some choline-ester splitting enzymes. If the enzyme is inhibited during runs, the velocity is expected to fall off; the extent to which this occurs, however, may not necessarily be such as is characteristic of a first-order reaction. Also this case seems to hold for a group of ChE reactions in fairly wide ranges of enzyme and substrate concentrations. In still other cases, the affinity for the enzyme may be so low that the reaction velocity decreases remarkably with time. This falling off may not be characteristic of a first-order reaction; if k_1 is constant, this may be regarded as a limiting case. At high substrate concentration the velocity may fall off to such a degree that k_1 falls off slowly. With lowering initial ester concentration the falling off is less and at a definite substrate concentration the reaction may revert to a first-order reaction. Such a case is the hydrolysis of BzCh by guinea-pig liver (Table 28). The system *Helix* blood—ACh is an extreme case of high enzyme affinity, the system guinea-pig liver—BzCh that of low enzyme affinity.

Almost the same series of enzyme-substrate systems is obtained by comparing the rise of k_1 from the highest to the lowest sub-

TABLE 30. *Characteristics of Some Typical Cholinesterase Reactions*
Horse serum, human erythrocytes, dog brain, *Helix* blood, dart sac,
guinea-pig liver.

| Characteristics | | Enzyme | Substrate |
|--|------------|---|----------------------------|
| k_0 constant (at least at fairly high substrate concentrations) | | <i>Helix</i> blood
Dart sac
Erythrocytes
Brain | ACh |
| | | Serum | BzCh |
| k_0 falls off and k_1 rises (even at very high substrate concentrations) | | Serum | ACh
ASaCh
TB |
| k_1 constant | | In some cases, but not characteristic of any reaction | |
| k_1 falls off (even at fairly high substrate concentrations) | | Erythrocytes
<i>Helix</i> blood
Dart sac? | MeCh
ASaCh |
| | | Liver | BzCh
ASaCh
ASa
TB |
| Rise of k_1 (enzymic) from the highest to the lowest substrate concentration (Sol. No. 1→Sol. No. 6) | < 25 | Liver | BzCh |
| | 25—100 | Serum
Dart sac | ACh |
| | 100—400 | <i>Helix</i> blood | ACh |
| | ≥ 400 | Erythrocytes
Brain | ACh |
| k_0 and/or b_{50} higher/lower at high than at low substrate concentration | Higher | Liver | BzCh |
| | | Serum
Dart sac | ACh |
| | Lower | Erythrocytes
Brain
<i>Helix</i> blood | ACh |

strate concentration. In the case of high enzyme affinity k_1 rises more than 400 times, when this affinity is low the corresponding rise is only about 15 times. Moreover, the cases of high enzyme affinities show lower reaction velocities at high substrate concentrations than at low ones. Enzymes of low affinities, on the other hand, have higher activities at high substrate concentrations than at low concentrations. More details about this relationship between enzyme activity and substrate concentration will be discussed in Chapter IX.

CHAPTER VIII

CHOLINESTERASE ACTIVITY AS FUNCTION OF ENZYME CONCENTRATION

Enzyme reactions give usually direct proportionality between reaction rate and enzyme concentration. Such a relationship was also found in the reactions studied in this investigation, provided that the enzyme concentration was not too high. Previous papers dealing with this relationship are, for example, those by ABDON and UVNÄS (1937), AUGUSTINSSON (1944), and BOELL and SHEN (1944).

At low enzyme concentration, the proportionality was always obvious, that is, when the substrate was continually in excess (Fig. 5). With increasing enzyme concentration a point was reached when no excess of substrate was present. When the enzyme concentration was increased further, the reaction rate would not be expected to change. The author is disposed to suppose that this was the case in the example 4 (Fig. 5). Moreover, there may not be any direct proportionality between reaction rate and enzyme concentration, if the enzyme preparation contains impurities which retard the action of the enzyme. In such cases, the reaction rate decreases gradually with increase in enzyme concentration. Such facts also may explain the deviation from proportionality (example 1). In most cases, however, it was difficult to draw conclusions as to whether the deviation was due to one or the other of these factors. As mentioned above the majority of reactions studied has shown direct proportionality between reaction rate and enzyme concentration.

The results reported in Chapter VI were obtained in experiments with enzyme concentrations chosen in such a way that the amount of CO_2 evolved during 30 minutes was 100—200 μl . These conditions were arranged in order to obtain a straight line relationship between the volume of CO_2 evolved and time of reaction. In most cases, it was necessary to dilute the original enzyme preparations in order to obtain a suitable concentration. The b_{30} values obtained with these diluted solutions have been recalculated for 100 μl . blood (plasma) or 100 mg. tissue. In the experiments with horse plasma, for example, the plasma was diluted with R_{30} , 1 ml. in 8 ml. R_{30} . Such a solution had the activity

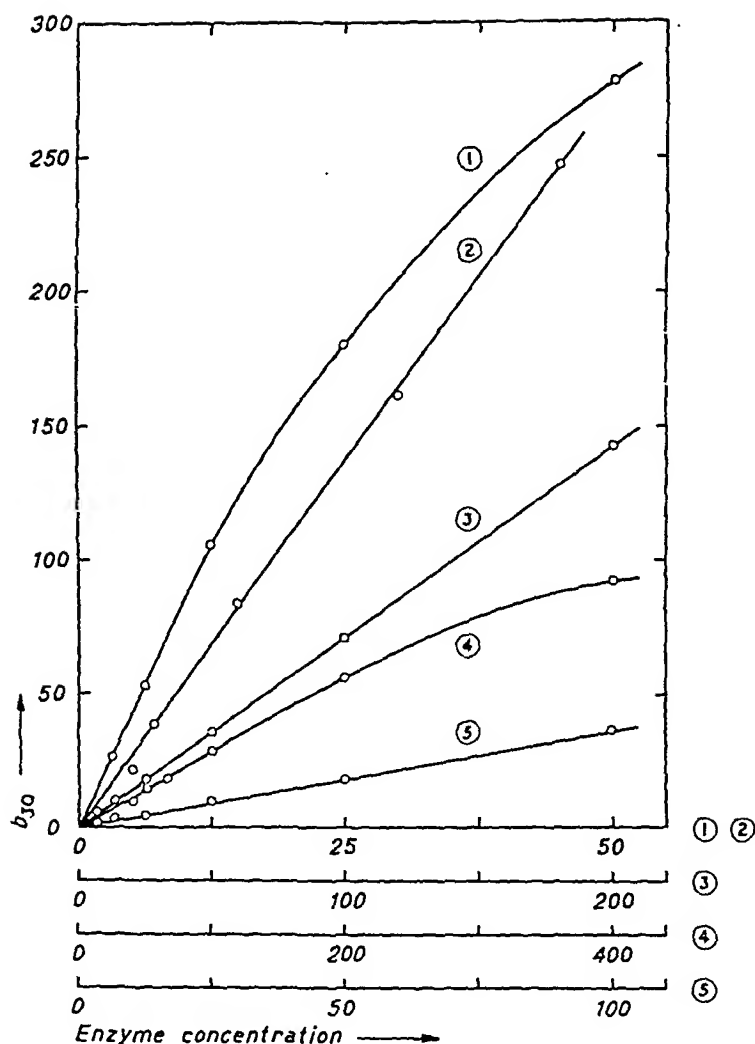


Fig. 5. Rate of enzymic hydrolysis as function of enzyme concentration.

1. ASaCh — guinea-pig liver (mg.). Data from Table 22.
2. ACh — horse serum (μ l. CO_2 in 60 min.).
3. BzCh — human plasma (μ l.).
4. ACh — horse erythrocytes (μ l. haemolysate). Data from Table 22.
5. ASa — guinea-pig intestine (mg.).

$b_{30} = 171.5$ towards ACh. The volume of enzyme preparation used was 400 μ l., corresponding to 50 μ l. plasma; hence, for 100 μ l. plasma, b_{30} was 343. Cow plasma was used undiluted; b_{30} was found to be 20 (for 400 μ l. plasma), that is 5 for 100 μ l. This has been the general principle of calculating the values in Tables 10 and 16. The same enzyme preparation was used for all substrates except in the case of great differences in b_{30} for various substrates (e.g., *Spirographis* blood). This arrangement was based on the

assumption of a direct proportionality between activity and enzyme concentration. As pointed out above, however, this proportionality was not always obvious, but the experiments were carried out in order to obtain an idea of the enzyme activity in each case and to form the basis for more advanced studies. Therefore the values listed in Tables 10 and 16 are not to be regarded as absolute.

CHAPTER IX

CHOLINESTERASE ACTIVITY AS FUNCTION OF SUBSTRATE CONCENTRATION

A. INTRODUCTION

The study of the activity-substrate concentration relationship has contributed greatly to our knowledge of the different types of ACh-hydrolysing enzymes. This relationship was used as one of the first arguments for differentiating between two different types of ChE. Thus the specific ChE is said to be inhibited by excess of substrate, which is not the case with the other type, the non-specific ChE (p. 19).

Before research had gone so far, several investigations were carried out in order to find out how the reaction velocity is influenced by the concentration of the substrate. The widely accepted theory of MICHAELIS and MENTEN (1913) has also been applied to the ACh-ChE system. The enzyme studied was the non-specific ChE (blood serum) in all cases. The dissociation constant (K_S) of the supposed intermediate compound between ChE and ACh was first determined by GLICK (1937 a) who found $K_S = 1.1 \times 10^{-3}$ (human serum). According to EADIE (1942) this constant is 1.7×10^{-3} (dog serum), to WRIGHT and SABINE (1943) 0.26×10^{-3} (dog serum) and 1.2×10^{-3} (human serum), and to GOLDSTEIN (1944) 1.25×10^{-3} (dog serum). The constants have been determined also for the enzyme complexes of arsenocholine and butyrylcholine (ROEPKE, 1937).

Since the observation by ALLES and HAWES (1940, 1944) that the ChE activity of red blood cells is depressed by excess of ACh which is not the case with the serum activity, more studies were performed on the activity-substrate concentration relationship of ChE. These studies have been reviewed above (p. 20). ACh only has been employed as substrate, except in an investigation by AUGUSTINSSON (1946) on the ChE of *Helix* blood. In this case the hydrolysis was depressed by excess of substrate using ACh, but not when MeCh was employed. This observation gave rise to more detailed studies on the activity-

substrate concentration relationships for a variety of enzyme-substrate combinations, the results of which will be discussed in this Chapter.

As is pointed out above (p. 20), MENDEL's group indicated that this relationship is not a criterion by which ACh-splitting enzymes can be differentiated. "The shape of the activity-substrate concentration curve cannot serve as a basis for distinguishing specific from non-specific cholinesterase" (HAWKINS & MENDEL, 1946). As regards the optimum substrate (ACh) concentration, contradicting results have been obtained by various authors. Thus ALLES and HAWES (1940, 1944) found that the erythrocyte ChE displays its optimum activity at ACh concentrations of pS 4.6 (pS = $-\log$ molar concentration). A similar value has been obtained by MENDEL and RUDNEY (1943, 1945) with the brain ChE in the absence of any salts other than NaHCO_3 . These results contrast with the findings of ZELLER and BISSEGGER (1943), NACHMANSOHN and ROTHENBERG (1945), and AUGUSTINSSON (1946). The pS values at which optimum activity is displayed by the erythrocyte ChE were found by these authors to be 2.35, 2.25, and 2.6 respectively; the values for the brain enzyme were 2.4, 2.0, and 2.5 respectively. In the author's opinion the differences between the high values of pS_{opt} obtained by the former groups of workers and the low ones by the latter may be due to the different experimental conditions and to the methods used.

These disagreements have been discussed by MENDEL and RUDNEY (1945) in a paper dealing with the effects of certain salts on the specific ChE (brain, erythrocytes). It was found that the relationship between ChE activity and substrate concentration was changed when salts, especially potassium chloride, were added to the medium. KCl in a concentration of 0.16 M caused a shift of the optimum activity of the enzyme (brain) from pS 3.60 to pS 2.52. Moreover, these authors found that KCl reduced the activity at low and increased it at higher concentrations, that is, the new optima corresponded to higher activities than in the absence of any salt (except NaHCO_3). This fact was said to bring "to light the cause of the discrepancy". In another paper of HAWKINS and MENDEL (1946), clupeine was found to alter the activity-substrate concentration relationship of the specific ChE (brain) in such a way that the enzyme displayed its maximum activity at high substrate concentration. The original relationship between enzyme activity and substrate concentration could then be restored by adding gum arabic to the clupeine-treated enzyme (cf. MENDEL & RUDNEY, 1944 b).

These observations are very interesting. It is important, however, to re-examine the actions of KCl and protamine in order to state whether the specific ChE of various sources behave according to the findings of MENDEL's group.

In the present investigation, the activity-substrate concentration curves were determined for a variety of enzyme-substrate combinations. The determinations were carried out in a medium

containing various substances, such as physostigmine, potassium chloride, clupeine, and gum arabic; in some cases lithium chloride was tested. The effect of choline on ChE activity has also been studied and will be discussed in Chapter X.

Physostigmine which is said to inhibit ChE, both the specific and non-specific enzyme, was used in order to compare the action of this drug on different enzyme-substrate systems, previously not studied. Potassium chloride (and lithium chloride) were employed in the light of MENDEL's observations and remembering that potassium escapes from cells which are stimulated. The strongly basic clupeine of the protamine group was expected to change the affinity of quaternary ammonium bases for the enzyme in such a way as was described by MENDEL. For the same reason, the effect of the negatively charged gum arabic was studied.

It has been shown in preliminary experiments with horse serum, horse erythrocytes, and dog brain, that Ca^{2+} (10^{-3} and 10^{-2} M respectively) does not alter the activity-substrate concentration relationships in any case.

As regards the selection of enzyme preparations, those have been examined which were expected to give the most characteristic patterns. Thus the following preparations were employed: blood serum, erythrocytes, brain, guinea-pig liver, cow kidney, *Sepia* "liver", *Helix* blood, and the dart sac of *Helix*. The methods of determinations are described above (Chapter III). In the side bulb 0.20 ml. of the enzyme solution was mixed with 0.20 ml. of a solution of the substance, whose influence on the ChE activity was to be studied. The concentrations of the substrates in each of the six solutions used are listed in Table 9 (p. 48—49), those of the substances used for influencing the enzyme activity in Table 7 (p. 40). In all cases the initial velocities were measured and the enzyme activities expressed by b_{30} .

B. THEORY

1. No Inhibition by Excess of Substrate

a) No Inhibitor Present

The generally accepted theory of MICHAELIS and MENTEN (1913) postulates that the enzyme combines with the substrate to form an unstable enzyme-substrate complex which then breaks down

in enzyme and reaction products. Let the enzyme (ChE in this case) be represented by E, the substrate (ACh) by S, and the intermediate complex by ES, the dissociation constant of which is K_s . At equilibrium we may write the mass law expression:

$$\frac{[S] ([E]_{\text{tot}} - [ES])}{[ES]} = K_s \quad (2)$$

supposing that the free molar concentration of the substrate, [S], is very much greater than $[E]_{\text{tot}}$ (= total enzyme concentration), and equal to the total (initial) substrate concentration. The rate of destruction of the substrate, v , that is the observed enzyme activity, is proportional to the concentration of the complex ES. Thus, $v = k[ES]$, where k is the velocity constant for the destruction of the substrate. In the presence of a large excess of substrate when all the enzyme is converted into ES, the reaction velocity will attain a limiting value, $V_{\text{max}} = k[E]_{\text{tot}}$. Hence, after substituting for v and V_{max} in Equation (2) and rearranging,

$$v = \frac{V_{\text{max}} [S]}{[S] + K_s} \quad (3)$$

According to this formula K_s is equal to the substrate concentration when the velocity v is one half the limiting velocity V_{max} .

The relationship between v and [S] will be shown graphically by plotting the velocity, v , expressed in $\mu\text{l. CO}_2$ per 30 minutes (b_{30}), against pS. The curves obtained, when no inhibition by excess of substrate is prevailing, are familiar dissociation curves.

The results are characterised by the two constants V_{max} and pK_s which are determined graphically, using the inverted Equation (3):

$$v + K_s \frac{v}{[S]} = V_{\text{max}} \quad (4)$$

Plotting v against $v/[S]$, a straight line is obtained, the intercepts of which are V_{max} and V_{max}/K_s on the v and $v/[S]$ axis respectively. In the cases of familiar S-shaped curves and of no inhibition by excess of substrate, approximately straight lines are usually obtained. This indicates that ChE actually combines with its substrate to form a complex. When the reaction velocities are very low, say in the presence of physostigmine in ChE reactions, no accurate values of V_{max} and K_s (K'_s)¹ have been

¹ K'_s symbolises the apparent dissociation constant when a constant concentration of inhibitor is present.

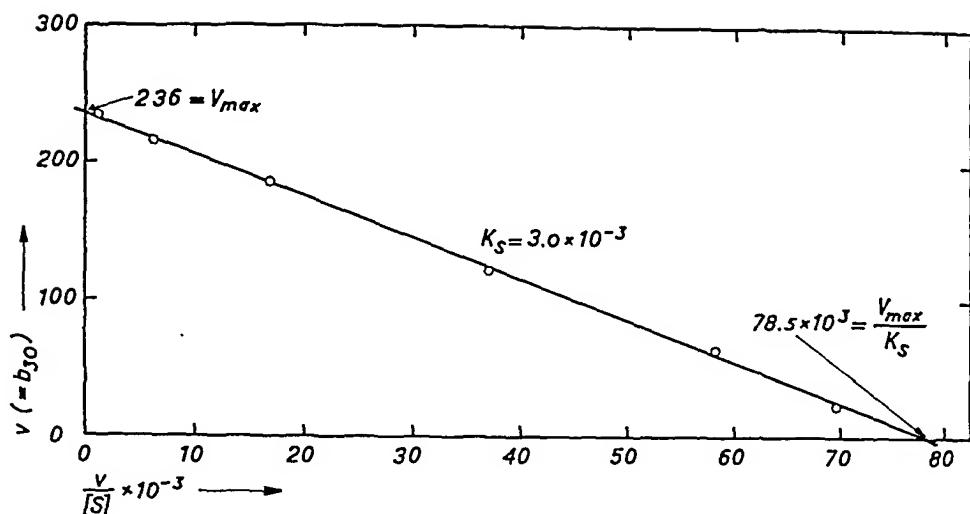


Fig. 6. Graphical analysis of the enzymic hydrolysis of ACh by horse serum ChE at various substrate concentrations and in the presence of gum arabic. Cf. Fig. 8, curve 5.

obtained. An example of the graphical procedure is given by Fig. 6. K_s may also be obtained by extrapolation on the activity-pS curve for $v = \frac{1}{2}V_{max}$.

The values of V_{max} and K_s have been used, as was said, in order to characterise numerically the differences when the same enzyme or various enzymes attack different substrates or the same substrate, also when the actions of certain substances on the enzyme activity were studied using K'_s instead of K_s . It will be remembered that such quantitative differences depend both on differences in the maximum velocity (V_{max}) of the hydrolysis occurring at infinite substrate concentration and in the affinity of the substrate for the enzyme (K_s). A low affinity is indicated by a high value of K_s (low value of pK_s), and *vice versa*. Moreover, consider an enzyme to catalyse the hydrolysis of two esters. If the enzyme concentration in both cases is the same, a constant ratio of the V_{max} values will be obtained from one preparation to another.

b) Inhibitor Present

Two enzymes may be identical if they behave similarly with respect to added substances. The reaction velocity v may be reduced equally at all substrate concentrations. In such a case K'_s is equal to K_s and the inhibition regarded as non-competitive. If the reduction is not the same at high and low substrate

concentrations, K'_s is not equal to K_s . At competitive inhibition, $K'_s > K_s$ that is, when the inhibition is greater at low than at high substrate concentrations, and a greater substrate concentration is necessary in order to saturate the enzyme.

Consider the reactions $E + S \rightleftharpoons ES$ and $E + I \rightleftharpoons EI$ and let K_s and K_I represent the dissociation constants of the complexes ES and EI respectively, and v' and v the reaction velocities in the presence of the inhibitor I and in its absence respectively. We have then

$$v' = \frac{V_{\max} [S]}{[S] + K_s + [I]K_s/K_I} = \frac{v}{1 + [I] K_s/K_I ([S] + K_s)} \quad (5)$$

where $[I]$ is the inhibitor concentration, supposed to be much greater than $[E]_{\text{tot}} (= [E] + [ES] + [EI])$. Rearranging the two expressions of v' ,

$$\frac{1}{v'} = \frac{1}{V_{\max}} + \frac{1}{V_{\max} [S]} \left(K_s + \frac{[I] K_s}{K_I} \right) \quad (6)$$

and

$$\frac{v}{v'} = 1 + [I] \left(\frac{K_s}{K_I ([S] + K_s)} \right) \quad (7)$$

According to (6), a plot of $1/v'$ against $1/[S]$ at constant concentrations of enzyme and inhibitor will give a straight line, the intercept of which on the $1/v'$ axis is $1/V_{\max}$. In the case of competitive inhibition, $1/V_{\max}$ is the same with and without inhibitor, in non-competitive inhibition it is not. This is the graphical procedure of LINEWEAVER and BURK (1934), applied to some cases in the present investigation (Chapter X). The value of the apparent dissociation constant K'_s is obtained by the same graphical analysis as that of K_s (Equation 4, Fig. 6). K_I may then be calculated as follows:

$$K_I = \frac{[I]}{\frac{K'_s}{K_s} - 1} \quad (8)$$

Also a plot of v/v' against $[I]$ gives a straight line (Equation 7) in the cases of competitive inhibition and constant concentrations of enzyme and substrate. This is the experimental result in some cases, reported in Chapter X. The intercept on the v/v' axis is 1, irrespective of substrate and rate of hydrolysis, which differs greatly from substrate to substrate in many cases.

The MICHAELIS-MENTEN formulation is based on the assumption that the enzyme concentration is so low, compared with the concentrations of substrate and inhibitor, that it can be neglected. More general equations have been presented by STRAUS and GOLDSTEIN (1943) and GOLDSTEIN (1944) who made allowance for the possibilities that the concentration of enzyme centres (serum ChE) is not extremely low and constant, and therefore may not be neglected. The present interpretation has not taken into consideration these authors' "zone behavior of enzymes", but are mainly based on the derivations made by MICHAELIS and MENTEN (1913), HALDANE (1930), and LINEWEAVER and BURK (1934).

2. Inhibition by Excess of Substrate

a) No Inhibitor Present

It is pointed out previously in this paper and shown also by experiments that the activity of the specific ChE is depressed by excess of substrate. Such an inhibition by strong substrate concentrations has been observed with other enzymes such as the liver esterases, oxygenase, catalase (for references, see HALDANE, 1930, and LINEWEAVER & BURK, 1934). It may be accounted for in terms of the following theory brought forward by HALDANE. This theory is in agreement with the experimental results presented below.

HALDANE suggested that a complex of the enzyme with two molecules of the substrate, ES_2 , is formed at high substrate concentrations. This complex is incapable of the breakdown yielding acid and alcohol. Let K_{S_1} and K_{S_2} represent the dissociation constants of the compounds ES and ES_2 in the reaction $E + S \rightleftharpoons ES$ and $ES + S \rightleftharpoons ES_2$ respectively. In the same way as above, we have

$$K_{S_1} = \frac{[S]([E]_{\text{tot}} - [ES] - [ES_2])}{[ES]} \quad \text{and} \quad K_{S_2} = \frac{[S][ES]}{[ES_2]} \quad (9 \text{ a, b})$$

Then, after eliminating $[ES_2]$ and introducing the velocity terms v and V_{max} ,

$$v = \frac{V_{\text{max}}[S]}{[S] + K_{S_1} + [S]^2/K_{S_2}} \quad (10)$$

This equation will give a symmetric bell-shaped curve when the velocity, v , is plotted against the logarithm of the substrate concentration, pS . As far as the author is aware, only a few enzyme

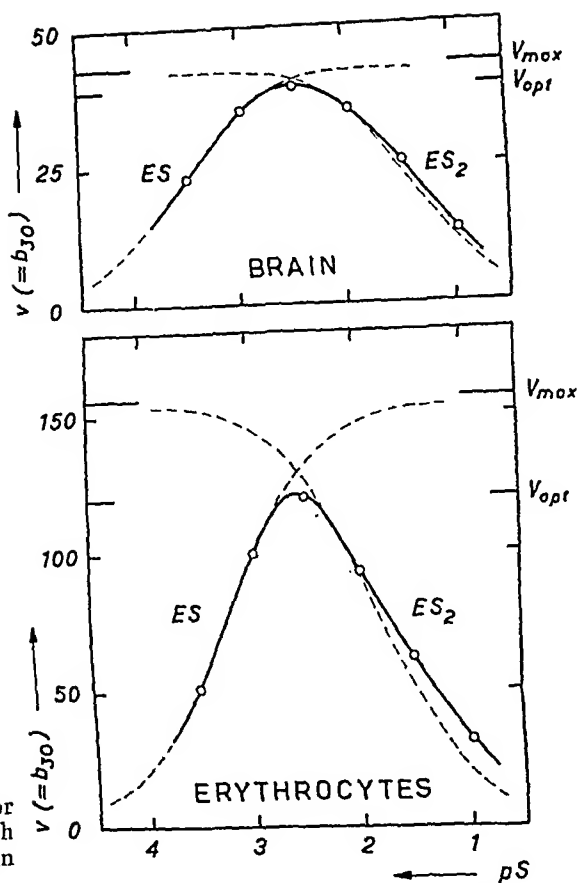


Fig. 7. Activity-pS curves for the enzymic hydrolysis of ACh by erythrocytes (cow) and brain (dog).

| | V_{\max} | V_{opt} | pS_{opt} | pK_{S_1} | pK_{S_2} | pS_1 | pS_2 |
|---------------------------------|------------|------------------|--------------------------|--------------------------|--------------------------|---------------|---------------|
| Erythrocytes | | | | | | | |
| Exper. data from Fig. 11, I ... | — | 122 | 2.55 | 3.2 | 1.75 | 3.4 | 1.45 |
| Calculated values | 156 | 130 | 2.5 | » | 1.8 | » | 1.6 |
| Brain | | | | | | | |
| Exper. data from Fig. 13 | — | 39 | 2.45 | 3.55 | 1.3 | 3.6 | 1.3 |
| Calculated values | 43 | 41 | 2.45 | » | 1.4 | » | 1.35 |

reactions have hitherto given evidence for such a symmetry and, in the words of MYRBÄCK (1940), "Diese Fällen ... dürften sehr selten sein". The data obtained with the specific ChE, however, show in many cases that the HALDANE interpretation is very well fitted to the reactions of this enzyme.

The reaction velocity represented by Equation (10) gives an optimum, V_{opt} , when $[S] = \sqrt{K_{\text{S}_1} K_{\text{S}_2}}$ or $\text{pS}_{\text{opt}} = \frac{1}{2}(\text{pK}_{\text{S}_1} + \text{pK}_{\text{S}_2})$. If ES_2 were not formed, the maximum velocity of the hydrolysis would be V_{\max} (Equation 3), evaluated according to the graphical method described above (Equation 4), which also gives K_{S_1} . In this way V_{\max} and K_{S_1} were evaluated for the hydrolysis of ACh by erythrocyte and brain ChE, the data of which are found in Fig. 7. The dotted curves represent the simple MICHAELIS-

MENTEN equations for the reactions $E + S \rightleftharpoons ES$ and $ES + S \rightleftharpoons ES_2$ if these were proceeding independently of each other. For instance, in the case of erythrocyte ChE activity,

$$v = \frac{156}{1 + 0.000631/[S]} \quad \text{and} \quad v = \frac{156}{1 + [S]/0.0159}$$

The fulldrawn lines are the experimentally found ones and agree well with the theoretical curves, hence indicating that the suggestion of HALDANE satisfactorily explains the facts.

For $v = \frac{1}{2} V_{\max}$ the values of K_{S_1} and K_{S_2} may be obtained and from these values we obtain the optimum substrate concentration. Because of the symmetry of the curve, all pairs of points with equal co-ordinates of the v axis give the same sum of the co-ordinates of the pS axis. For the sake of convenience, the author has used those pS values which are easily found on the experimentally found curve when $v = \frac{1}{2} V_{\text{opt}}$. The two values thus obtained are symbolised by pS_1 and pS_2 respectively. As V_{opt} is always less than V_{\max} , the value of $pS_1 > pK_{S_1}$ and that of $pS_2 < pK_{S_2}$, which is demonstrated by the values in Fig. 7. If $pS_{\text{opt}} = \frac{1}{2} (pS_1 + pS_2)$ or half the sum of other pairs of pS values (and of pK_{S_1} and pK_{S_2}), the curve is really symmetric.

b) Inhibitor Present

The equations presented above may be extended to systems containing competitive inhibitors, the full discussion of which is very complicated. Consider a change in the first dissociation constant K_{S_1} (not in K_{S_2}) and in addition the absence of any enzyme-inhibitor complexes except EI , the dissociation constant of which is K_I . We have then, using the same symbols as above,

$$\frac{v}{v'} = 1 + [I] \left(\frac{K_{S_1}}{K_I ([S] + K_{S_1} + [S]^2/K_{S_2})} \right) \quad (11)$$

Hence, at constant concentrations of enzyme and substrate, a straight line should be expected in the plot of v/v' against $[I]$. This is the experimental result in the cases of low substrate concentrations when pS_{opt} is not yet reached and therefore the complex ES_2 is not formed (Equation 7). When the substrate concentration is above the optimum, the situation is more complex (Chapter X, Fig. 21). In such cases straight lines have not been obtained, due to the fact that in the presence of choline the

optimum substrate concentration is raised. This situation will be discussed more fully below (p. 135).

C. EXPERIMENTAL RESULTS

1. Blood Serum

a) *Horse serum*. In the determination of the relationship between enzyme activity and substrate concentration for horse-serum ChE, a purified preparation of Cf \approx 3 000 (p. 24) was employed, obtained by fractional ammonium-sulphate precipitation (p. 42). The preparation was diluted with distilled water to give an adequate enzyme concentration for the manometric method. This enzyme solution was used in all experiments, the results of which are found in Fig. 8.

Confirming previous findings, the system ChE-ACh gave an activity-pS curve of the familiar shape of a dissociation curve. The value of K_s was determined graphically according to Equation (4). This procedure gave $pK_s = 2.5$, which is somewhat lower than GLICK's (1937 a) 2.95, EADIE's (1942) 2.77, and GOLDSTEIN's (1944) 2.90; these values were all obtained with crude enzyme preparations. When horse plasma was used, instead of a purified ChE preparation, somewhat higher values of pK_s have been obtained. The mean of three such measurements was 2.83 which agrees well with those of the other authors.

Physostigmine (3.63×10^{-6} M) inhibited the enzymic hydrolysis of ACh to a great extent at all substrate concentrations. K'_s was very much greater than K_s and the inhibition is regarded as competitive. Potassium chloride (0.1 M) had a very slight action on the esterase activity. Clupeine reduced the reaction velocity; in 1.10×10^{-2} -M ACh-chloride solution 0.05 per cent clupeine inhibited the activity 82 per cent. When the substrate concentration was 10 times higher, the inhibition was 42 per cent. Because of that pK_s was greater than pK'_s . Such a "lowering of pK_s " in the presence of clupeine, as will be seen, is characteristic of nearly all ACh-hydrolysing enzymes, no matter which type of ChE may be used. In the presence of 0.05 per cent gum arabic, the reaction velocities were increased slightly at about the same degree at all substrate concentrations and therefore $pK_s = pK'_s$.

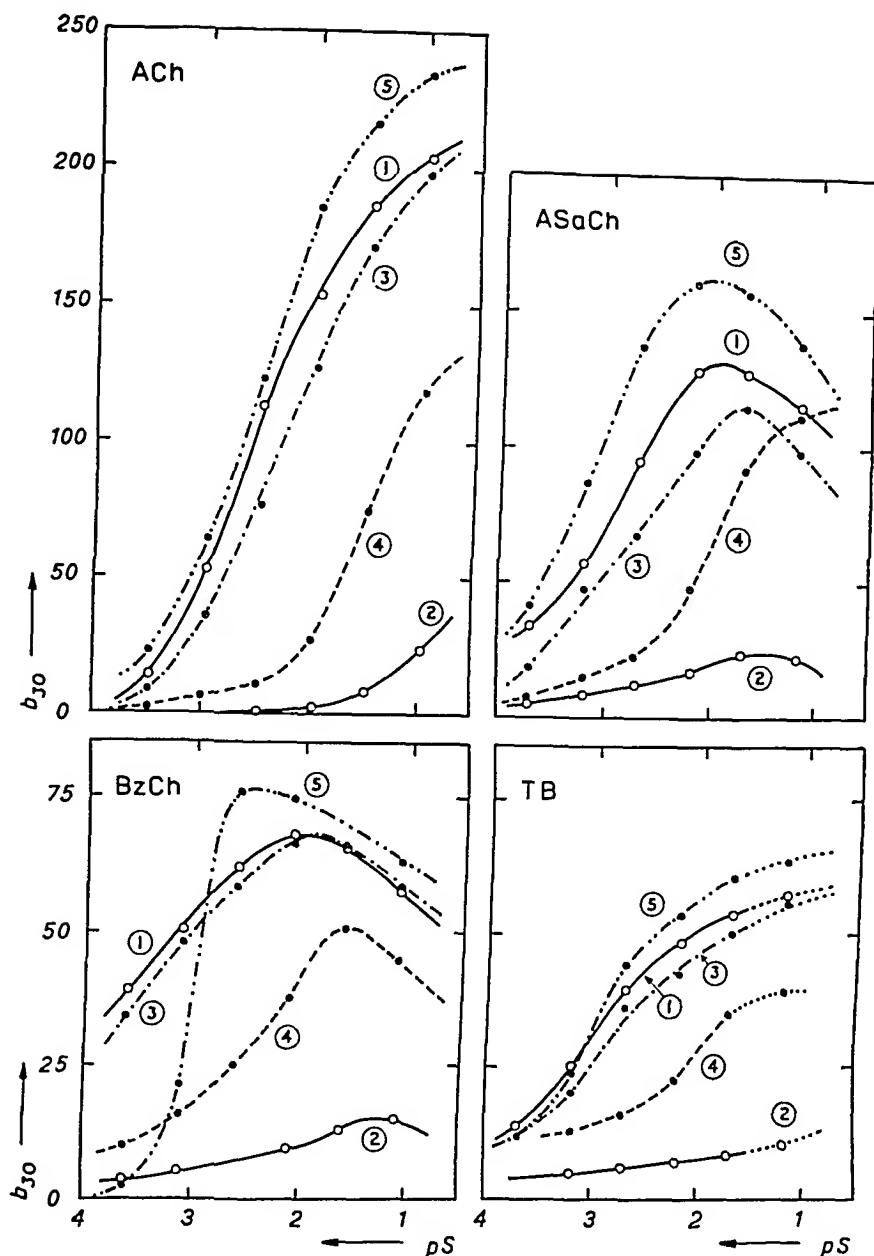


Fig. 8. Activity-pS curves for the enzymic hydrolysis of ACh, BzCh, ASaCh, and TB by a purified ChE preparation from horse serum.

| | ACh | | BzCh | | | ASaCh | | | TB |
|---------------------|------------|--------------|------------------|-------------------|--------|------------------|-------------------|--------|----------------|
| | V_{\max} | $pK_S^{(1)}$ | V_{opt} | pS_{opt} | pS_1 | V_{opt} | pS_{opt} | pS_1 | |
| 1. Control..... | 210 | 2.5 | 68 | 2.0 | 3.8 | 131 | 2.0 | 3.05 | S-shaped curve |
| 2. Physostigmine. ? | ? | ? | 16 | 1.3 | ? | 24 | 1.6 | ? | Inhibition |
| 3. KCl..... | 202 | 2.3 | 67 | 1.9 | 3.65 | 115 | 1.75 | 2.9 | No action? |
| 4. Clupeine..... | 140 | 1.6 | 51 | 1.6 | 2.6 | 126 | — | 2.0 | Inhibition |
| 5. Gum arabic... | 236 | 2.5 | 77 | 2.5 | 3.0 | 162 | 2.1 | 3.25 | No action? |

(cf. Fig. 6)

The activity-pS curve for the enzymic hydrolysis of BzCh had quite another shape than in the case of ACh. High substrate concentrations depressed the activity towards BzCh; the enzyme displayed its optimum activity at pS_{opt} 2.0. In a cruder enzyme preparation, pS_{opt} was somewhat higher, *i.e.*, 2.3. It is not justifiable, however, to draw conclusions only from this observation as to whether the destructions of ACh and BzCh by serum are catalysed by the same or distinct enzymes. In spite of the differences in activity-substrate concentration relationships, the substances added had similar effects on the hydrolysis of BzCh as on that of ACh. These substances did not alter the shape of the activity-pS curve. Thus physostigmine reduced the reaction velocity remarkably at all BzCh concentrations; pS_{opt} was changed from 2.0 to 1.3. Potassium chloride had hardly any action at all. Clupeine displaced the optimum substrate concentration to higher concentrations, gum arabic did so in the opposite direction. The action of gum arabic was such that the enzyme activity was inhibited at low substrate concentrations.

The experiments with ASaCh gave almost the same patterns as with BzCh. The hydrolysis was decreased by excess of substrate and both substrates gave the same values of pS_{opt} . Physostigmine inhibited strongly. Clupeine did so, too, and in its presence a higher optimum (maximum?) substrate concentration was obtained. Gum arabic activated.

The values obtained in the enzymic hydrolysis of TB were not very certain, especially at high substrate concentrations. It was obvious, however, that the reaction velocities were not decreased by excess of TB, that physostigmine inhibited, that potassium chloride and gum arabic had no or very slight action, and that clupeine inhibited the enzyme activity. All these facts agree with those found in the experiments with choline esters. This is a further proof for the identity of the esterases catalysing the hydrolysis of choline esters and TB.

b) *Fowl plasma*. It was found in preliminary experiments that fowl plasma splits MeCh at a higher rate than BzCh, and hence the enzyme might be regarded as a specific ChE according to MENDEL's definition. The enzyme differs, however, from the specific ChE of erythrocytes and brain in the fact that the hydrolysis of ACh is not depressed by excess of substrate. The activity-pS curves for the enzymic hydrolysis of ACh, MeCh, and ASaCh are

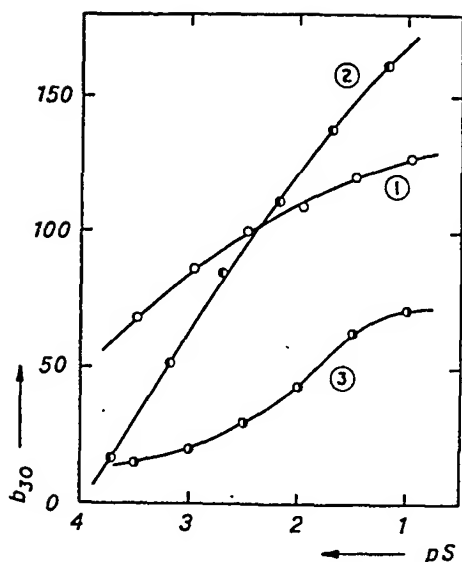


Fig. 9. Activity- pS curves for the enzymic hydrolysis of ACh, MeCh, and ASaCh by fowl plasma (400 μ l.).

1. ACh; 2. ASaCh; 3. MeCh.

shown in Fig. 9. No further experiments have been carried out with this preparation, and it is difficult to draw any conclusion as to the type of ChE present in cock plasma. It will be noted that the ACh and ASaCh curves are not of the pure S-shaped type. Perhaps a mixture of choline-ester splitting enzymes is present, but this question may be open for further discussion.

c) *Shark (Scyllium) plasma* hydrolyses ACh at a very low rate (Table 10), in contradistinction to the blood cells which are completely inactive. The plasma enzyme resembled the specific ChE in the depressant effect of high ACh concentrations on the activity (Fig. 10). The optimum concentrations of ACh and ASaCh corresponded to pS_{opt} 2.7 and 2.2 respectively. It may be observed that at high substrate concentrations ASaCh was

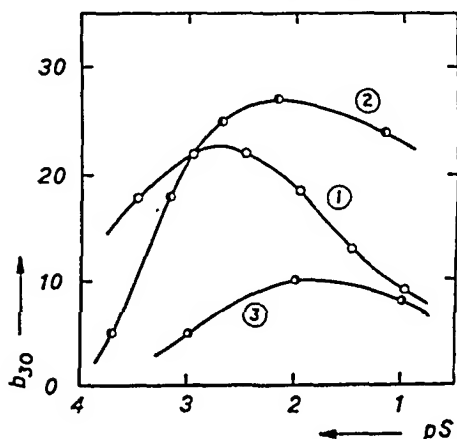


Fig. 10. Activity- pS curves for the enzymic hydrolysis of ACh, ASaCh, and MeCh by shark plasma (200 μ l.). 25.0°C.

| | V_{opt} | pS_{opt} | pS_1 | pS_2 |
|-------------|-----------|-------------|-------------|--------|
| 1. ACh..... | 23 | 2.7 | ≈ 4 | 1.3 |
| 2. ASaCh .. | 27 | 2.2 | 3.2 | ? |
| 3. MeCh ... | 10 | ≈ 2 | ? | ? |

split at a higher rate than ACh which, on the other hand, was hydrolysed at a higher rate than ASaCh at low substrate concentrations. Also the hydrolysis of MeCh seemed to be depressed by high substrate concentrations.

2. Erythrocytes

a) *Cow erythrocytes*. The hydrolysis of ACh through the action of erythrocytes was decreased by high concentrations of substrate (Fig. 11). The activity-pS curve is bell-shaped and its symmetry is almost complete, thus indicating that the HALDANE interpretation is well fitted to this reaction (see also Fig. 7). It is obvious that the activity-substrate concentration relationships of serum- and erythrocyte-ChE respectively are very distinct. But the influences of various substances on both enzymes were of the same kind. Thus physostigmine inhibited the enzyme activity to the same great extent at all ACh concentrations. Clupeine caused a shift of the optimum substrate concentration to higher concentrations (change of pS_{opt} from 2.55 to 1.85). This is very characteristic and significantly established in nearly all reactions of ChE. This action of clupeine is irrespective of the choline ester used, the enzyme preparation may be crude or purified (see ACh I and II in Fig. 11). At higher substrate concentrations the right-hand side of the bell-shaped curve approached the curve when no clupeine was present.

Gum arabic slightly activated the erythrocyte ChE at medium substrate concentrations and was without effect at high ones, resulting in a small shift of pS_{opt} to higher values.

An activation by potassium chloride (0.1 M) was also observed at all substrate concentrations and pS_{opt} was not altered. This was in sharp contrast to the results of MENDEL and RUDNEY (1945); according to these authors, KCl causes a shift of the optimum activity to lower pS values. Such a shift of pS_{opt} has not been obtained in any case, no matter which substrate and enzyme preparation (erythrocyte or brain, crude or pure preparations) were used. Lithium chloride (not shown in Fig. 11) behaved in the same way as the potassium salt. In this respect attention will be called to the following. It is not correct to make manometric readings 2 minutes after mixing substrate and enzyme (as was done by MENDEL). At this time, thermal equilibrium is not reached. Moreover, it is impossible to get accurate values of

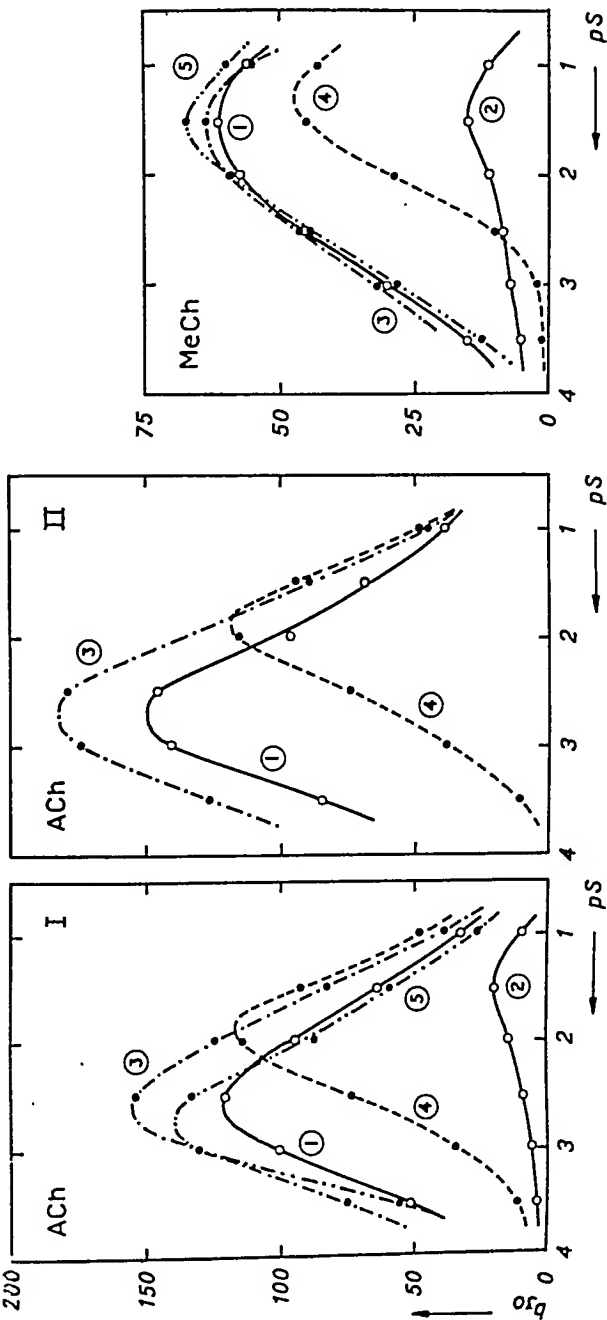


Fig. 11. Activity-pS curves for the enzymic hydrolysis of ACh by haemolysate (I) and a purified ChE preparation (II) of cow erythrocytes, and of MeCh by haemolysate.

| | ACh I | | | | ACh II | | | | MeCh | | | |
|-----------------------------|-----------|------------|--------|--------|-----------|------------|---------------|--------|-----------|------------|---------------|---------------|
| | V_{opt} | pS_{opt} | pS_1 | pS_2 | V_{opt} | pS_{opt} | pS_1 | pS_2 | V_{opt} | pS_{opt} | pS_1 | pS_2 |
| 1. Control (cf. Fig. 7) ... | 122 | 2.55 | 3.4 | 1.45 | 149 | 2.7 | 3.55 | 1.6 | 61 | 1.5 | 1.55 | 2.95 |
| 2. Physostigmine..... | 20 | 1.5 | 2.25 | 1.01 | — | — | — | — | 15 | 1.5 | ≈ 2.5 | ≈ 2.5 |
| 3. KCl..... | 155 | 2.6 | 3.5 | 1.45 | 182 | 2.7 | ≈ 3.7 | 1.55 | 63 | 1.5 | 3.0 | 3.0 |
| 4. Clupcine..... | 116 | 1.85 | 2.65 | 1.15 | 118 | 1.85 | 2.65 | 1.1 | 47 | 1.3 | 2.15 | 2.15 |
| 5. Gum arabic..... | 139 | 2.7 | 3.40 | 1.70 | — | — | — | — | 67 | 1.5 | 1.85 | 2.85 |

the amount of CO_2 evolved when the ACh concentration is very low. The lowest ACh-chloride concentration used in the present investigation has been 3.3×10^{-4} M (solution No. 6); at such a concentration under the experimental conditions 15 μl . CO_2 is evolved at complete hydrolysis. The extrapolated b_{∞} values for the (6)-solutions were in fact not very exact. At still lower substrate concentrations, as those used by MENDEL, this exactness will be reduced further. Therefore, the results of MENDEL's school must be regarded as dubious.

The activity-pS curve for the enzymic hydrolysis of MeCh showed a lower value of pS_{opt} ; at very high substrate concentrations the activity was decreased. Because of that, MeCh at high concentration was hydrolysed at a higher rate than ACh at the same high concentration. When the substrate concentration in both cases was 0.1 M, the ratio between the reaction velocities for ACh and MeCh was 0.57, in 0.001-M solutions, on the other hand, this ratio was 3.34. In comparison of the hydrolysis of ACh and of other esters, therefore, it is important to consider these differences, previously not taken notice of. It may well explain the findings by RICHARDS JR. and CUTKOMP (1945) and by TOBIAS, KOLLROS and SAVIT (1946) that insect nervous system is more active on MeCh than on ACh (cf. also AUGUSTINSSON, 1946 c). In fact, the molar concentrations of the substrates used by the former authors were high (about 0.1). It may also explain the result of ALLES and HAWES (1940) that "the red blood cell enzyme is more active towards acetyl- β -methylcholine than towards acetylcholine".

Physostigmine inhibited the destruction of MeCh by erythrocytes, but pS_{opt} was not altered. Clupeine had a similar action: inhibition and no or slight change of pS_{opt} . The MeCh curves were similar to the BzCh and ASaCh curves found in experiments with serum ChE (Fig. 8). The activity-pS curve for the system MeCh-erythrocytes was not influenced by potassium chloride or gum arabic.

The activity-pS curve for the hydrolysis of ASaCh, shown in Fig. 12, has quite another shape than in the cases of ACh or MeCh. The rate of reaction was not decreased by high substrate concentrations and a familiar S-shaped curve was obtained. For comparison the corresponding ACh curve is also shown in Fig. 12. The ratio between the ACh and ASaCh hydrolysis in 0.1-M solutions was 0.221, in 0.001-M solutions ten times higher or

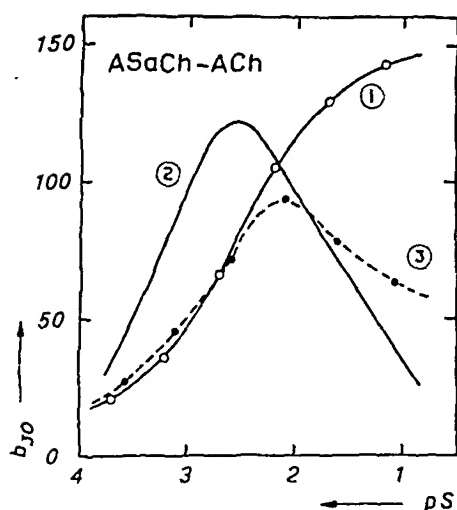


Fig. 12. Activity-pS curves for the enzymic hydrolysis of ASaCh by haemolysate of cow erythrocytes compared with that of ACh and the hydrolysis of a mixture of ACh and ASaCh.

| | V_{max} | V_{opt} | pS_{opt} | pS_1 | pS_2 | pK_S |
|---------------------------|-----------|-----------|------------|--------|--------|--------|
| 1. ASaCh | 147 | | — | — | — | 2.6 |
| 2. ACh (Fig. 11, I) | | 122 | 2.55 | 3.4 | 1.45 | — |
| 3. ASaCh + ACh | | 93 | 2.1 | 3.05 | ? | — |

2.17. Hence, when the hydrolysis of ACh is compared with that of ASaCh, the substrate concentration must be considered. The activity-pS curve (Fig. 12) has also been determined for the hydrolysis of a mixture of ACh and ASaCh; the concentrations of the two substrates in the mixture were each half of those when the substrates were studied separately.

It is obvious that ACh and ASaCh compete for the same active group(s) of the enzyme molecule. But the mechanisms of the two reactions are not the same at high and low substrate concentrations. At high concentrations ACh forms the complex ES_2 , which is incapable of breakdown (p. 102). At the same time ACh inhibits the hydrolysis of ASaCh. This means that the second centre of the ChE molecule, combining with a second ACh molecule, also takes part in the breakdown of ASaCh. The situation is very complex, but it is supposed that at high ASaCh concentrations the acetyl-salicylic linkage only is split, the breakdown of the choline-ester linkage does not occur, no more than it does in the case of ACh at high concentrations. At low substrate concentrations, on the other hand, both ester linkages in ASaCh are supposed to split at the same time. Proofs for this hypothesis have been offered by experiments where the hydrolysis of ASaCh was allowed to proceed for a long time to give complete destruction. Table 31 shows the results of two experiments with high

TABLE 31. *Total Hydrolysis of ASaCh by Cow Erythrocytes (100 μ l.)*

| Time
(min.) | | μ l. CO ₂ evolved | |
|----------------------------|-----------|----------------------------------|----------|
| | | ASaCh(2) | ASaCh(4) |
| 8 | | 60 | 36 |
| 24 | | 151 | 61 |
| 60 | | 315 | 71.5 |
| 140 | | 508 | 93 |
| 240 | | 630 | 99.5 |
| 339 | | 699 | 109 |
| 1 330 | | 899 | 154 |
| 1 345 | | 899 | 155 |
| 1 360 | | 899 | 155 |
| Calcul.
values <i>a</i> | one link | 896 | 89.5 |
| | two links | 1 792 | 179 |

and low ASaCh concentrations respectively. As expected, only one of the ester linkages was split enzymically at high substrate concentration (ASaCh 2) and this linkage was the acetyl-ester one, as will be shown in Chapter X. Moreover, at low ASaCh concentration (ASaCh 4) both ester linkages were broken, for the hydrolysis proceeded till the amount of CO₂ liberated, calculated for the breakdown of two ester linkages, was reached approximately.

Erythrocytes hydrolysed TB at a low rate. The enzyme responsible for this reaction is not the same as that catalysing the hydrolysis of choline esters. It was established that the TB hydrolysis was neither inhibited by excess of substrate, nor by physostigmine (b_{50} 25 for TB(3) with and without inhibitor). Nor did clupeine inhibit the esterase activity towards TB.

b) *Horse erythrocytes*. Almost the same results have been obtained with the blood-cell ChE of horse. The value of pS_{opt} was 2.5 (for cow erythrocytes 2.55) and was the same for crude and pure preparations. No details are given in this case because of the similarities with the corresponding cow enzyme.

3. Brain

The results obtained with dog brain extracts (Fig. 13) were much the same as those described for the enzyme activity of red blood cells. All data favour the supposition that the ChE of erythrocytes and that of brain are identical.

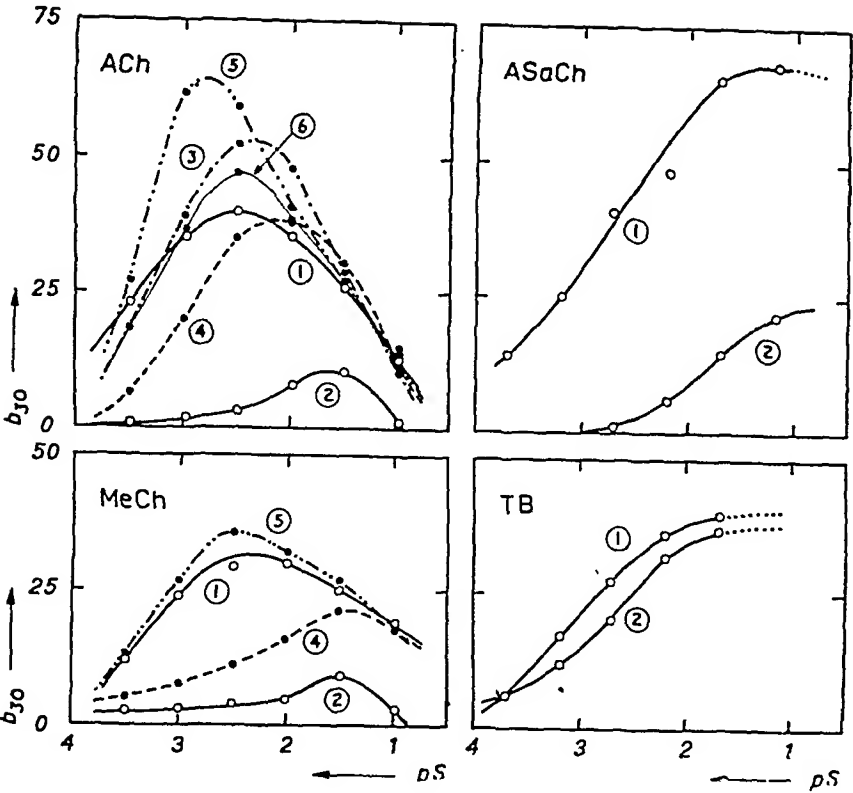


Fig. 13. Activity-pS curves for the enzymic hydrolysis of ACh, MeCh, ASaCh, and TB by dog brain (50 mg.).

| | ACh | | | | MeCh | | | |
|------------------------------|-----------|------------|--------|--------|-----------|------------|--------|--------|
| | V_{opt} | pS_{opt} | pS_1 | pS_2 | V_{opt} | pS_{opt} | pS_1 | pS_2 |
| 1. Control (cf. Fig. 7) | 40 | 2.45 | 3.6 | 1.3 | 32 | 2.4 | 3.35 | 0.85 |
| 2. Physostigmine..... | 11 | 1.6 | 2.2 | 1.1 | 9 | 1.5 | ≈ 2 | 1.1 |
| 3. KCl | 53 | 2.35 | 3.3 | 1.4 | — | — | — | — |
| 4. Clupeine | 39 | 2.1 | 3.0 | 1.1 | 21 | 1.4 | 2.6 | ≈ 0.5 |
| 5. Gum arabic | 64 | 2.8 | 3.4 | 1.7 | 36 | 2.5 | 3.35 | 0.9 |
| 6. LiCl | 47 | 2.4 | 3.3 | 1.35 | — | — | — | — |

| | ASaCh | | TB |
|-----------------------|-----------|---------|-------------------|
| | V_{max} | pK'_s | |
| 1. Control | 68 | 2.8 | S-shaped curve |
| 2. Physostigmine..... | 25 | 1.8 | Slight inhibition |

The brain ChE-activity was inhibited by high ACh concentrations and the bell-shaped activity-pS curve was symmetric (cf. Fig. 7), indicating that HALDANE's theory of the complex ES_2 is true for this enzyme. The value of pS_{opt} was 2.45, very near the value of the erythrocyte enzyme (2.55). Physostigmine prevented greatly the destruction of ACh at all substrate concentrations and pS_{opt} was lowered to 1.6 in the same way as was found for the blood-cell ChE. The characteristic shift in pS_{opt}

to lower value in the presence of clupeine was also observed. Gum arabic activated the brain ChE, also found with other types of ChE; pS_{opt} seemed to shift a little towards lower substrate concentrations. The value of V_{opt} was increased by potassium chloride and the same effect was observed with lithium chloride. pS_{opt} , however, was not altered, in contrast to MENDEL's observation (p. 97).

The activity-pS curves for the enzymic hydrolysis of MeCh were much the same as those obtained with ACh. In contrast to the erythrocyte ChE, the values of pS_{opt} were the same in both cases. The added substances had the same general influences on the hydrolysis: inhibition by physostigmine, shift of optimum substrate concentration to higher concentration in the presence of clupeine, and a small activation by gum arabic.

ASaCh gave quite a different activity-pS curve than ACh and MeCh. The enzyme activity was not depressed by excess of substrate. In spite of this, the same enzyme might split ACh and ASaCh. This appeared from the potent inhibiting action of physostigmine. Most probably, the same interpretation reported above for the erythrocyte ChE is also applicable to the brain enzyme.

In all likelihood, the hydrolysis of TB by brain extracts is not catalysed by the enzyme splitting choline esters. Physostigmine had no or very slight inhibiting action on this reaction. The rate of hydrolysis was not depressed by high substrate concentrations.

4. Liver

a) *Guinea-pig liver*. It was shown in Chapter VI that guinea-pig liver splits ACh at a very low rate, in contrast to BzCh. SAWYER (1945) and BLASCHKO *et al.* (1947 a, b) suggested a "benzoylcholine esterase" present in high concentration and not identical with the "pseudo-ChE". The experimental results obtained by the present author are shown in Fig. 14. The experiments with added substances are not included for BzCh, ASaCh, and TB, as the curves were close to those obtained when a second compound was not added.

The low rate of hydrolysis of ACh was not decreased by high substrate concentrations. Physostigmine inhibited, but not as strongly as was expected for a ChE. Clupeine was also an inhibitor, but the action was quite weak. Gum arabic had no effect

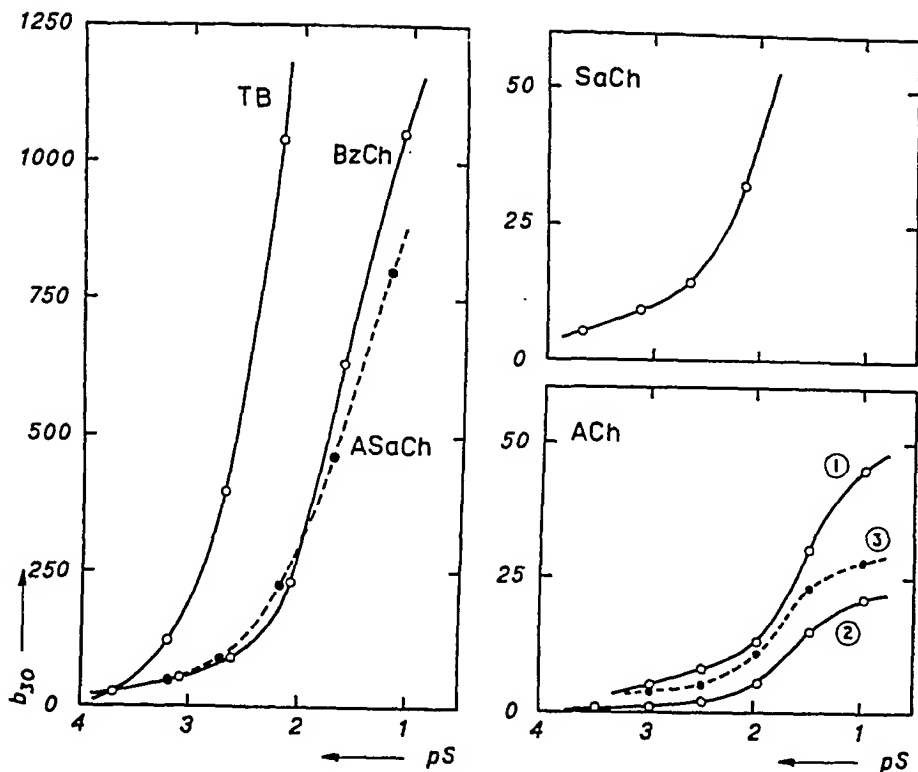


Fig. 14. Activity-pS curves for the enzymic hydrolysis of ACh, SaCh, BzCh, ASaCh, and TB by guinea-pig liver. Extrapolated b_{30} values in each case.

| Substrate | BzCh | ASaCh | TB | SaCh | ACh | 1. Control
2. Physostigmine
3. Clupeine |
|------------|------|-------|----|------|-----|---|
| mg. tissue | 50 | 50 | 25 | 100 | 100 | |

on this reaction (not shown in Fig. 14). ACh may be destroyed by the same enzyme that acts upon BzCh, but definite conclusions cannot be given.

Guinea-pig liver is remarkable for its very high activity towards BzCh, not found with any other material. The activity-pS curve was not the same as other curves for this substrate. Inhibition by high substrate concentrations, found for instance in experiments with the non-specific ChE of blood serum, was not observed. Physostigmine (10^{-6} M) inhibited only slightly (17 per cent). Clupeine and gum arabic activated a little or had no effect at all. Potassium chloride did not affect the activity. All these findings indicate that the enzyme is distinct from ChE (the specific as well as the non-specific one). Some of the results obtained in experiments with added compounds are found in Table 33 (p. 140).

The liver splits SaCh at a higher rate than ACh (Fig. 14).

It is supposed that the same enzyme splits this choline ester and BzCh. The two reactions seem to have the same value of pK_s .

The hydrolysis of ASaCh (Fig. 14) showed much the same properties as that of BzCh. It gave the same type of activity- pS curve and was inhibited by physostigmine to about 20 per cent (Table 33). Clupeine activated slightly and so did gum arabic. Potassium chloride did not alter the activity-substrate concentration relationship. All data obtained indicate that BzCh and ASaCh are split by the same enzyme, the properties of which are distinct from those of other choline-ester splitting enzymes.

TB was destroyed by guinea-pig liver at a very high rate which increased rapidly with the increase of substrate concentration. Physostigmine had no action and the same held for clupeine. Gum arabic activated slightly at all substrate concentrations. In this connection it is interesting to note that Fodor (1947) for some esters found an inhibiting action of gum arabic on the esterase activity of certain liver preparations and proposed the co-existence of two different esterases in liver juice.

b) *Fish liver*. In experiments with *Gadus* liver, the enzymic hydrolysis of ACh was found to be decreased by high ACh concentrations. pS_{opt} was about 2.5, that is, the same value obtained for the specific ChE of brain or erythrocytes. Also the *Labrus*-liver ChE was inhibited by high ACh concentrations. Experiments, carried out at 25°C, gave pS_{opt} 1.7. These data are to be regarded as preliminary as they are the only ones carried out with these tissues. Additional experiments must be performed in order to draw conclusions as to the type of ChE present in fish liver.

c) *Sepia "liver"*. As pointed out above, the experiments with *Sepia* "liver" were carried out at 25°C. The hydrolysis of ACh was depressed by high substrate concentrations (Fig. 15). The enzyme displayed its optimum activity at pS_{opt} 2.6 which was about the same found for the brain and erythrocyte enzymes. Physostigmine inhibited the activity strongly at all substrate concentrations. Clupeine had the same characteristic influence on the activity as was found for other choline-ester splitting enzymes. As seen in Fig. 15, clupeine inhibited the ChE activity at low ACh concentrations, but activated it at high concentrations; in a 1.1×10^{-2} -M ACh solution this protamine did not alter the activity at all. This shows how misleading the results

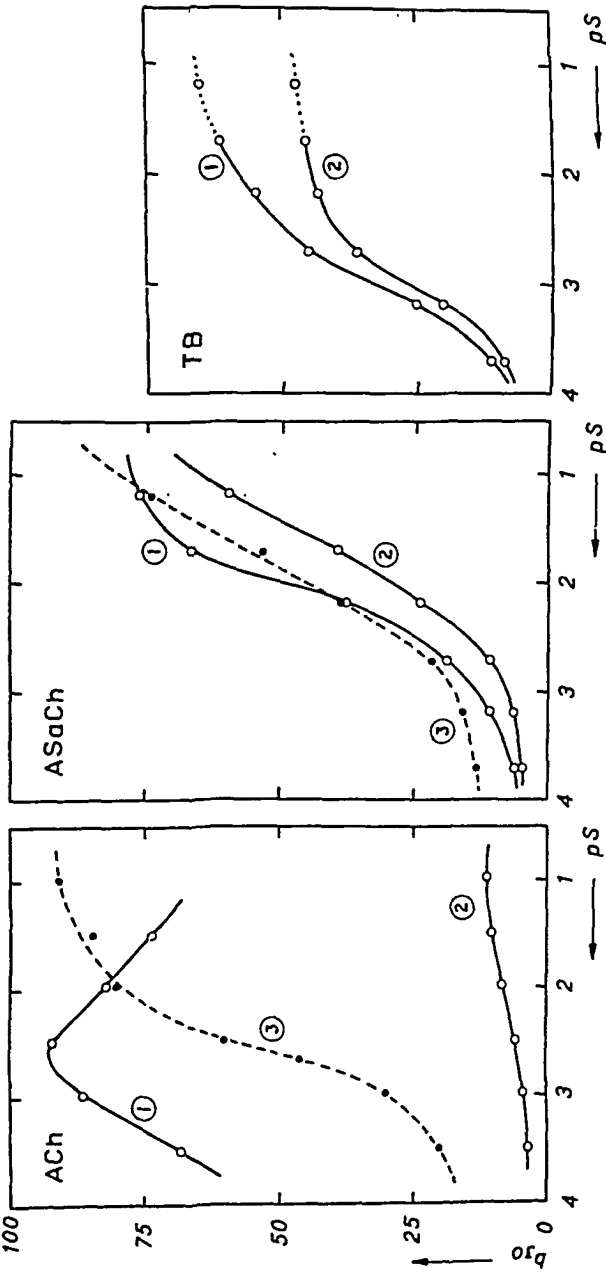


Fig. 15. Activity-pS curves for the enzymic hydrolysis of ACh, ASaCh, and TB by *Sepia* "liver", (50 mg.), 25.0° C.

| | ACh | | | | ASaCh | | | TB | |
|-----------------------|-----------|-----------|------------|--------------|-----------|--------------|--|-------------------|--|
| | V_{opt} | V_{max} | pS_{opt} | $pK_S^{(1)}$ | V_{max} | $pK_S^{(1)}$ | | | |
| 1. Control | 92 | | 2.6 | — | 84 | 2.1 | | S-shaped curve | |
| 2. Physostigmine..... | 11 | | ? | — | ≈75 | ≈1.8 | | Slight inhibition | |
| 3. Clupeine | | 92 | — | 2.65 | ≈90 | ≈2 | | | |

may be if the effect of a substance is measured at one substrate concentration arbitrarily chosen. This consideration resolves, most probably, many of the controversies on the effects of certain compounds on ChE activity, occurred in the literature (cf. Table 4).

It was found in the hydrolysis of ASaCh by erythrocytes that the activity-pS curve was a familiar dissociation curve, in contrast to the bell-shaped curve for the ACh hydrolysis. Similar observations have been made with *Sepia* "liver" (Fig. 15). But in the latter case, physostigmine inhibited only slightly the activity towards ASaCh and clupeine had scarcely any effect at all. The hydrolysis of ASaCh was much more like that of TB than of ACh. Thus, the TB reaction was also influenced only slightly by physostigmine.

These results justify to suppose that *Sepia* "liver" contains two distinct esterases, one acting upon ACh and one upon TB. No conclusion will be drawn as to which of these enzymes that splits ASaCh, possibly both are active on this ester. For further discussion the reader is referred to Chapter XII.

5. Kidney

a) *Cow kidney*. It is characteristic of the esterase activity of cow kidney, that, among choline esters, ASaCh is split at the highest rate. BzCh is hydrolysed at a low but relatively higher rate than ACh and MeCh. The activity-pS curve for the enzymic hydrolysis of ASaCh (Fig. 16) is a familiar dissociation curve. Possibly the esterase is a "salicylesterase" of the same type as has been found in *Spirographis* blood (AUGUSTINSSON, 1947). For comparison, the activity-pS curve for the hydrolysis of ASaCh by this polychaete blood is inserted in Fig. 16. The same pK_s (2.0) was found in both cases and physostigmine inhibited only slightly the two esterases.

TB gave the same type of activity-pS curve as was found in the experiments with guinea-pig liver. The rate of hydrolysis increased very rapidly with the increase in TB concentration.

b) *Guinea-pig kidney*. Similar results have been obtained with this tissue. ASaCh gave the same activity-pS curve with the same value of pK_s . Physostigmine did not inhibit the esterase which may be a "salicylesterase" with high activity towards ASa.

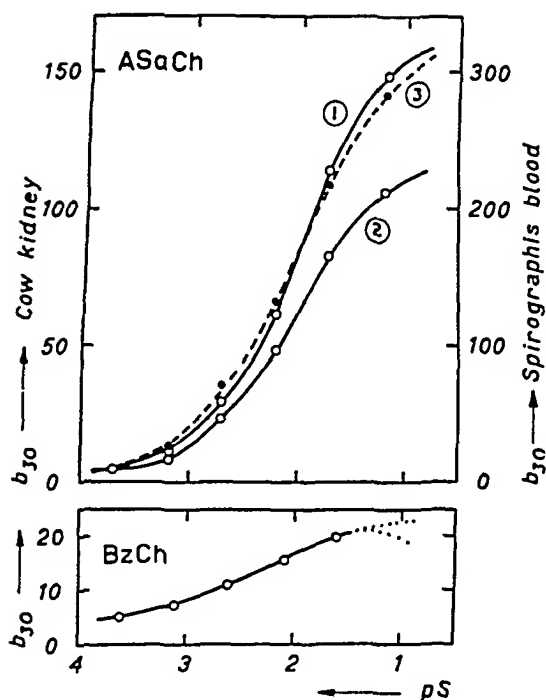


Fig. 16. Activity-pS curves for the enzymic hydrolysis of ASaCh and BzCh by cow kidney (50 mg.). Results obtained with *Spirographis* blood have been inserted for comparison (cf. AUGUSTINSSON, 1947).

| | | ASaCh | |
|---|---------------------|------------|--------------|
| | | V_{\max} | $pK_S^{(1)}$ |
| Cow kidney | 1. Control..... | 166 | 2.0 |
| | 2. Physostigmine... | 120 | 2.0 |
| <i>Spirographis</i> blood (50 μ l.) | 3. Control..... | 336 | 2.0 |

6. *Helix* Blood

In a previous paper (AUGUSTINSSON, 1946 c), the author studied the activity-substrate concentration relationship of the *Helix*-blood ChE. These studies have been extended and the results are shown in Figs. 17 and 18.

The enzyme displayed its optimum activity at pS_{opt} 2.7 when ACh was employed as substrate, which is much the same as that found for the brain and erythrocyte ChE. The same result was obtained with a purified ChE preparation (curve 5 in Fig. 17) of the snail blood. Physostigmine exerted strong inhibition at all substrate concentrations and pS_{opt} was lowered. In the presence of 0.1-M potassium chloride, the enzyme activity was changed only slightly. Lithium chloride (not shown in Fig. 17) had no effect (pS_{opt} 2.65 and no inhibition or activation). Clupeine showed the same characteristic effect as was found for the ChE

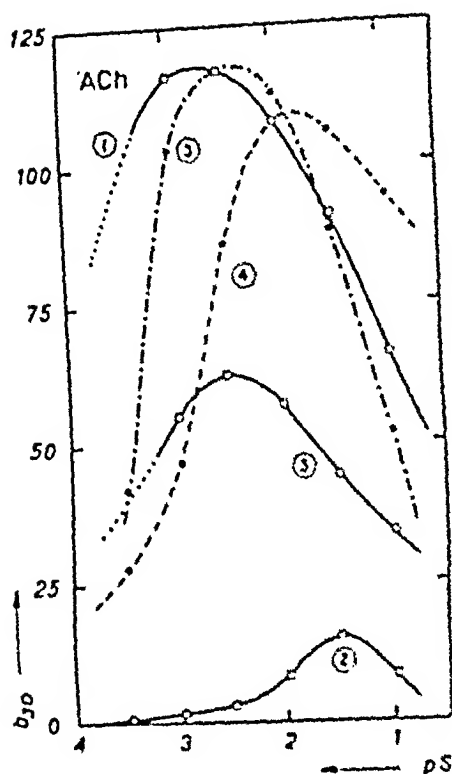


Fig. 17. Activity-pS curves for the enzymic hydrolysis of ACh by *Helix* blood (20 μ l.).

| | V_{opt} | pS_{opt} | pS_i | pS_i |
|--|-----------|------------|-------------|--------|
| 1. Control | 118 | 2.7 | ≈ 4 | 0.8 |
| 2. Physostigmine | 15 | 1.5 | 2.0 | 0.95 |
| 3. KCl | 118 | 2.3 | 3.35 | 1.1 |
| 4. Clupeine | 108 | 1.6 | 2.85 | ? |
| 5. A purified preparation (control)..... | 62 | 2.5 | ≈ 4 | 0.5 |

of other sources, that is, inhibition and change of pS_{opt} to lower values.

When MeCh was used as substrate, the enzyme displayed its optimum activity at a much higher substrate concentration (pS_{opt} 1.35) (Fig. 18). This agreed with the results found for the erythrocyte ChE. Clupeine inhibited at all substrate concentrations. Almost the same patterns were obtained with ASaCh and AAn, demonstrated in Fig. 18.

The activity-pS curve for the enzymic hydrolysis of TB had not the same shape as in the cases of choline esters (and AAn), and clupeine was without effect. Probably TB is split by a different enzyme in *Helix* blood from the one which acts upon choline esters. The concentration of such a tributyrinase, however, must be relatively low.

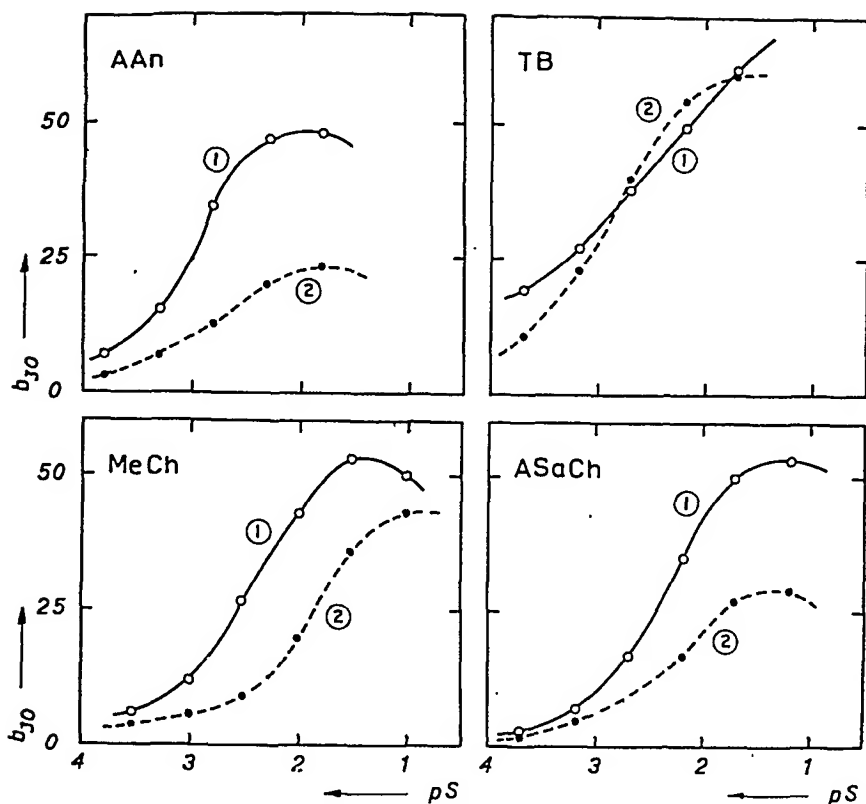


Fig. 18. Activity-pS curves for the enzymic hydrolysis of MeCh, ASaCh, AAn, and TB by *Helix* blood (20 μ l.).

| | MeCh | | | ASaCh | | | AAn | | | TB (200 μ l.) | |
|---------------|-----------|------------|-------------|-----------|------------|--------|-----------|------------|---------------|-------------------|---------------|
| | V_{opt} | pS_{opt} | pS_1 | V_{opt} | pS_{opt} | pS_1 | V_{opt} | pS_{opt} | pS_1 | V_{max} | $pK_S^{(1)}$ |
| 1. Control... | 54 | 1.35 | 2.5 | 54 | 1.35 | 2.4 | 48 | 2 | 3.0 | ≈ 80 | ≈ 2.6 |
| 2. Clupeine.. | 44 | ? | ≈ 2 | 30 | 1.4 | 2.3 | 24 | < 2 | ≈ 2.9 | 60 | 2.95 |

7. Dart Sac

In a previous paper (AUGUSTINSSON, 1946 c), the author pointed to the striking difference between the enzymes of *Helix* blood and the dart sac. It was shown for instance, that the ChE activity of the dart sac was not depressed by excess of substrate (ACh, MeCh, BzCh). These studies have been repeated and extended. The results are shown in Fig. 19.

It is characteristic of this ChE that it splits ACh at a very high rate, and MeCh and BzCh at relatively low rates. With respect to the activity-substrate concentration relationship, the enzyme is much more like the non-specific ChE of horse blood serum than the specific ChE of erythrocytes and brain. But no doubt, the dart-sac enzyme differs in some fundamental respects from

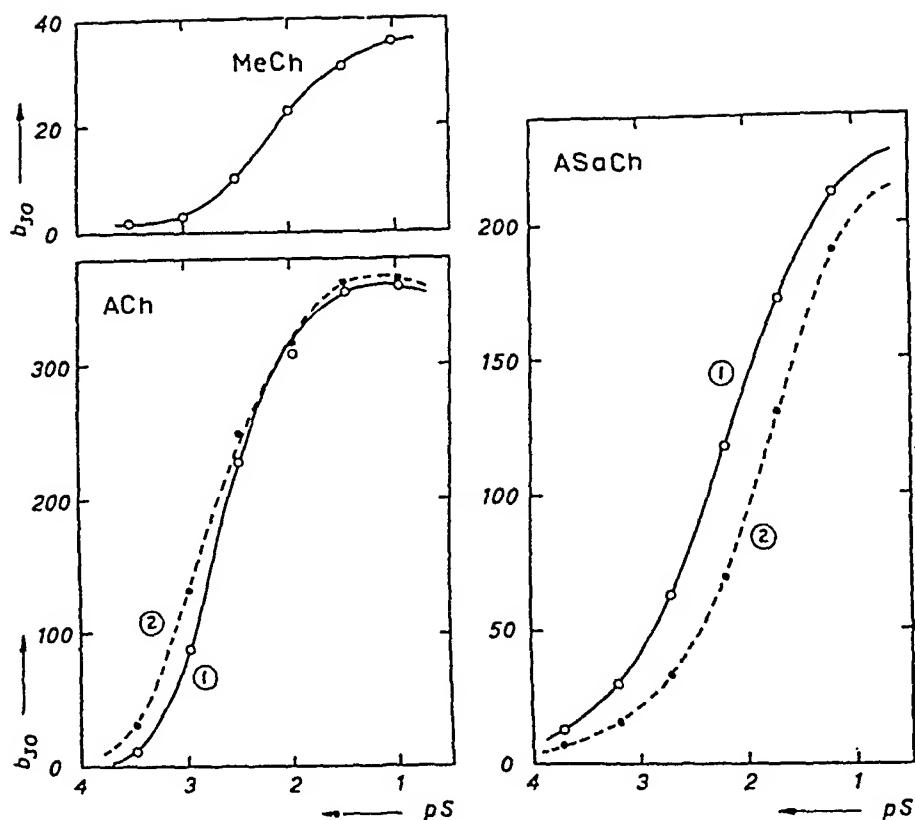


Fig. 19. Activity- pS curves for the enzymic hydrolysis of ACh, MeCh, and ASaCh by the dart sac (25 mg.).

| | ACh | | MeCh | | ASaCh | |
|-------------------|-----------|--------------|-----------|--------|-----------|--------------|
| | V_{max} | $pK_S^{(1)}$ | V_{max} | pK_S | V_{max} | $pK_S^{(1)}$ |
| 1. Control | 360 | 2.7 | 38 | 2.15 | 230 | 2.2 |
| 2. Clupeine | 363 | 2.75 | — | — | 218 | 1.9 |

the non-specific ChE. Thus, the enzyme displays its maximum (optimum?) activity at a lower ACh concentration than the serum enzyme. Clupeine had no or very little effect on the dart-sac enzyme. This stands in opposition to the characteristic effect of this protamine on other choline-ester splitting enzymes.

The low activity towards MeCh and BzCh is not depressed by excess of substrate; also this is in opposition to the properties of the serum enzyme. Striking differences of the two enzymes have also been observed in the hydrolysis of ASaCh (cf. Figs. 8 and 19). The activity- pS curve was a familiar dissociation curve in the case of the dart sac. The serum enzyme, on the other hand, gave a definite pS_{opt} at 2.0. Clupeine affected the dart-sac

enzyme weakly, in any case the characteristic change of the curve was not observed.

It is supposed that the same enzyme of the dart sac splits ACh, MeCh, and ASaCh. In Chapter XII further proofs will be offered for this assumption.

D. SUMMARY OF CHAPTER IX

The activity-substrate concentration relationships for choline-ester splitting enzymes from various sources have been studied, and the theory of MICHAELIS and MENTEN was applied to them. The inhibition by excess of substrate was interpreted in terms of HALDANE's theory, suggesting that a complex of the enzyme with two molecules of the substrate was formed. The action of some compounds was studied in this connection; the compounds were physostigmine, potassium chloride (in some cases lithium chloride), clupeine, and gum arabic.

A purified ChE preparation from horse serum gave a familiar dissociation curve when it acted upon ACh; K_s was 3.2×10^{-3} . The hydrolysis of BzCh and ASaCh was depressed by excess of substrate; the optimum substrate concentration in both cases was 1.0×10^{-2} M. The ChE activities of erythrocytes and brain were depressed by high ACh concentrations; optimum substrate concentrations were 2.8×10^{-3} M and 3.55×10^{-3} M respectively. Also the hydrolysis of MeCh was depressed by high substrate concentrations in both cases. ASaCh, on the other hand, gave familiar dissociation curves for both esterases. The enzymes of *Sepia* "liver" and *Helix* blood resembled much those of erythrocytes and brain in their actions on ACh; the enzymes displayed their optimum activities in 2.5×10^{-3} -M and 2.0×10^{-3} -M solutions respectively. Familiar dissociation curves were obtained in both cases in the hydrolysis of ASaCh. The activity-substrate concentration relationships for the choline-ester splitting enzymes of fowl and shark plasma have also been studied. Moreover the hydrolysis of BzCh and ASaCh by guinea-pig liver and that of ASaCh by cow kidney have been considered; in these cases familiar dissociation curves were obtained.

Physostigmine (3.63×10^{-5} M) inhibited strongly the ChE activity at all substrate concentrations. Strong inhibition was observed also in the hydrolysis of TB by horse plasma. The hydrolysis of BzCh and ASaCh by guinea-pig liver and cow

kidney were influenced only slightly by physostigmine, so also the destruction of ASaCh and TB by *Sepia* "liver". Physostigmine did not inactivate the tributyrinase activities of erythrocytes (cow) and brain (dog). The action of physostigmine on ChE seemed to be competitive in all cases of strong inhibition.

Potassium chloride (0.10 M) had no or very weak action on the ChE activity. It did not change the optimum substrate concentration for the enzymes of erythrocytes and brain.

Clupeine (0.05 per cent) had a characteristic effect on the ChE activity. It inhibited the enzyme competitively which means that the dissociation constant for the enzyme-substrate complex in the presence of clupeine has a higher value than in its absence. When the enzyme was inhibited by high substrate concentrations, clupeine inhibited only the enzyme activity at low substrate concentrations changing the optimum substrate concentration to higher values. Clupeine did not influence the esterases of guinea-pig liver and the dart sac.

Gum arabic (0.05 per cent) activated slightly the ChE activity in certain cases.

CHAPTER X

EFFECT OF CHOLINE ON CHOLINESTERASE ACTIVITY

A. INTRODUCTION

It was shown in Chapter VII that the reaction velocities in some cases decrease during the course of hydrolysis more than expected for a first-order reaction. This falling off of the reaction constants may be due in part to the inhibiting action of choline produced during the reaction.

The inhibiting action of choline on the enzymic hydrolysis of ACh by blood serum was first demonstrated by ROEPKE (1937) and later on studied by other authors (ZIFF, JAHN & RENSHAW, 1938; GAUTRELET & SCHEINER, 1939; AMMON, 1943; CASIER & DELAUNOIS, 1946). In all these cases blood serum was employed. Moreover, SÜLLMANN (1945) has stated that choline inhibits the hydrolysis of TB by blood serum, but not that by red blood cells.

Applying the theory of MICHAELIS and MENTEN, ROEPKE demonstrated that choline and some of its derivatives inhibit the ChE activity competitively. Assuming simultaneous adsorption of ACh and choline on the surface of ChE, ZIFF *et al.* derived an equation

for the relative affinities of choline derivatives for the enzyme surface and presented a method for measuring these affinities. This interpretation was based on the finding that the inverted value of the first-order reaction constant is a linear function of the choline concentration.

The theoretical interpretation in Chapter IX, based on the theory of MICHAELIS and MENTEN, has also pointed to such a relationship between reaction rate and concentration of a competitive inhibitor. In order to compare the action of choline on various reactions, the present author has plotted the ratio v/v' against the concentration of the inhibitor, in this case choline; v and v' symbolise the observed velocities of the hydrolysis, expressed by b_{30} , in the absence and presence of choline respectively. According to Equation (7) (p. 101) a straight line with the intercept "one" is obtained on the v/v' axis, if the inhibition is competitive. This is the experimental results for the hydrolysis of various esters by horse plasma and *Helix* blood (Fig. 20). In other cases, such straight lines have not been obtained (Fig. 21).

The effect of choline at various substrate concentrations have also been studied. The results are recorded as activity-pS curves (Figs. 22, 24, 26, 27, 28) and in some cases graphically analysed by the LINEWEAVER-BURK procedure (Figs. 23, 25, 29). At competitive inhibition, this is greater at low than at high substrate concentration (see Equation (6), p. 101).

When the enzyme activity is depressed by high substrate concentrations, it was considered in Chapter IX that an inhibitor I forms the complex EI with the enzyme E, thus disturbing the equilibrium for the complex ES. In the presence of such an inhibitor the optimum substrate concentration would be higher than in its absence. This is the experimental results for the enzymic hydrolysis of ACh by brain and erythrocytes (Figs. 24 and 26).

B. INHIBITION AS FUNCTION OF CHOLINE CONCENTRATION

The ChE activities of various tissues were studied in the presence of various concentrations of choline. The results are listed in Table 32 and some of them shown graphically in Figs. 20 and 21.

If choline exerted a purely competitive inhibition a straight line would be the result by plotting v/v' against the concentration of choline (according to Equation (7), p. 101). Such are the experi-

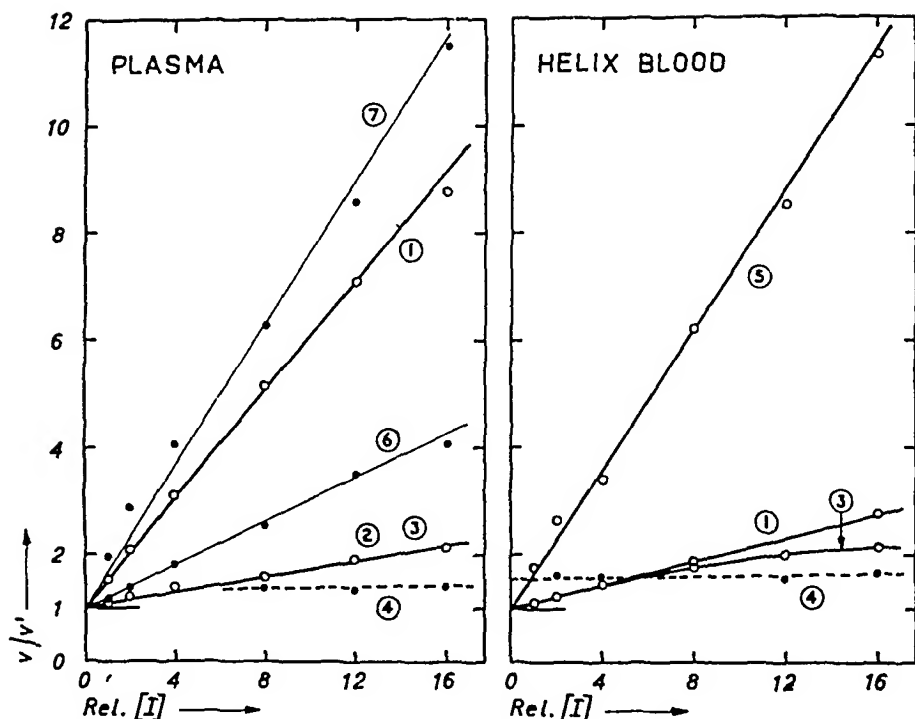


Fig. 20. Inhibition of ChE from horse plasma and *Helix* blood by choline.
Data from Table 32.

| Substrate | | Slope = $K_S/K_I([S] + K_S)$ | |
|-------------|-------------------------|------------------------------|--------------------|
| | | Plasma | <i>Helix</i> blood |
| 1. ACh(3) | 1.10×10^{-2} M | 57.3 * | 12.6 |
| 2. BzCh(3) | 8.22×10^{-3} M | 8.4 | — |
| 3. ASaCh(3) | 6.62×10^{-3} M | 8.4 | 10.5 |
| 4. TB(3) | 6.62×10^{-3} M | — | — |
| 5. AAn(3) | 5.28×10^{-3} M | — | 72.6 |
| 6. ACh(2) | 3.30×10^{-2} M | 22.3 * | — |
| 7. ACh(4) | 3.30×10^{-3} M | (75.4) | — |

* $K_S = 2.5 \times 10^{-3}$; $K_I = \begin{cases} 3.2 \times 10^{-3} \text{ (ACh3)} \\ 3.15 \times 10^{-3} \text{ (ACh2)} \end{cases}$; cf. Fig. 23.

mental results, found in Fig. 20. Thus choline inhibited competitively the hydrolysis of ACh, BzCh, and ASaCh by horse plasma. It inhibited much more strongly the destruction of ACh than it did those of BzCh and ASaCh. This means that the enzyme has a higher affinity for BzCh and ASaCh than for ACh. Much higher concentrations of choline are needed to give the same inhibition of the breakdown of BzCh than of ACh.

Choline inhibited only slightly the hydrolysis of TB by horse plasma and the inhibition was not competitive. Also SÜLLMANN (1945) has observed an inhibition of serum tributyrinase, the action of choline in his experiments being somewhat more potent.

TABLE 32. *Inhibition of ChE**v* and *v'* expres-

Relative choline conc.

Absolute » » $\times 10^2$ M.....

| Enzyme | | Substrate | v | Relative | |
|---------------------------------|---------------------------------|-----------|---------|----------|------|
| Preparation | μ l. mg.
per
2.00 ml. | | | 1 | |
| | | | | v' | v/v' |
| Horse plasma
(Fig. 20) | 50 | ACh(2) | 103 | 88 | 1.17 |
| | 100 | ACh(5) | 403 | 263 | 1.54 |
| | 50 | ACh(4) | 69 | 35 | 1.97 |
| | 100 | BzCh(3) | 161 | 145 | 1.11 |
| | 100 | ASaCh(3) | 269 | 247 | 1.09 |
| | 100 | TB(3) | (144) | (110) | 1.31 |
| Cow erythrocytes
(Fig. 21) | 100 | ACh(2) | 78 | 54 | 1.44 |
| | 100 | ACh(3)* | 120 | 77 | 1.56 |
| | 100 | ACh(4) | 130 | 81.5 | 1.59 |
| | 100 | ACh(5) | 102 | 39 | 2.62 |
| | 100 | MeCh(3) | 59 | 31 | 1.91 |
| | 100 | ASaCh(3)* | 122 | 51 | 2.39 |
| | 100 | TB(3) | 30 | — | — |
| Dog brain
(Fig. 21) | 100 | ACh(3) | 93 | 66 | 1.41 |
| | 100 | MeCh(3) | 44 | 26 | 1.69 |
| | 100 | ASaCh(3) | 102 | 61 | 1.67 |
| | 100 | TB(3) | 73 | 61 | 1.20 |
| Guinea-pig liver | 100 | BzCh(3) | (282.5) | (232.5) | 1.22 |
| | 100 | ASaCh(3) | (237) | — | — |
| | 100 | TB(3) | (425) | — | — |
| Cow liver | 50 | ASaCh(3) | (134) | (130.5) | 1.03 |
| <i>Helix</i> blood
(Fig. 20) | 10 | ACh(3) | 75 | 68 | 1.10 |
| | 10 | ASaCh(3) | 24 | 21 | 1.14 |
| | 20 | AAn(3) | 34 | 19 | 1.79 |
| | 200 | TB(3) | 55 | 46 | 1.20 |
| Dart sac | 25 | ACh(3) | 331 | 286 | 1.16 |
| | 25 | ASaCh(3) | 110 | 98 | 1.12 |
| | 25 | TB(3) | 38 | 38 | 1.00 |

*

| Rel. conc. | 6 | | 10 | | 14 | |
|------------|----|------|----|------|----|------|
| ACh(3) | 48 | 2.50 | 37 | 3.25 | 26 | 4.62 |
| ASaCh(3) | 18 | 6.78 | 15 | 8.13 | 12 | 10.2 |

It will be remembered that all results reported above argue that the same enzyme in horse serum splits TB and choline esters. The action of choline goes to prove that it cannot be the same

from Various Sources by Choline

sed in b_{30} .

| | | | | | |
|-------|------|------|------|-------|-------|
| 1 | 2 | 4 | 8 | 12 | 16 |
| 0.895 | 1.79 | 3.58 | 7.16 | 10.74 | 14.32 |

concentration of choline chloride

| 2 | | 4 | | 8 | | 12 | | 16 | |
|---------|--------|-------|--------|-------|--------|-------|--------|-------|--------|
| v' | v/v' | v' | v/v' | v' | v/v' | v' | v/v' | v' | v/v' |
| 73.5 | 1.40 | 55 | 1.87 | 40.5 | 2.54 | 29 | 3.56 | 25.5 | 4.04 |
| 195 | 2.07 | 130 | 3.10 | 78 | 5.17 | 57 | 7.08 | 46 | 8.78 |
| 24 | 2.88 | 17 | 4.06 | 11 | 6.27 | 8 | 8.6 | 6 | 11.5 |
| 132 | 1.22 | 120 | 1.34 | 105 | 1.54 | 84 | 1.92 | 79 | 2.04 |
| 224 | 1.20 | 185 | 1.45 | 164 | 1.64 | 139 | 1.93 | 122.5 | 2.19 |
| (113) | 1.28 | (103) | 1.40 | (102) | 1.41 | (105) | 1.37 | (103) | 1.40 |
| 49 | 1.59 | 41 | 1.90 | 37 | 2.11 | 33 | 2.36 | — | — |
| 64 | 1.88 | 50 | 2.40 | 43 | 2.79 | 38 | 3.16 | 18 | 6.67 |
| 60 | 2.17 | 43.5 | 2.99 | 27 | 4.81 | 21 | 6.19 | 16 | 8.13 |
| 19.5 | 5.23 | 11.5 | 8.87 | 7 | 14.6 | 5 | 20.4 | 2 | 51 |
| 21 | 2.81 | 13 | 4.54 | 10 | 5.90 | 8.5 | 6.94 | 6 | 9.84 |
| 34 | 3.59 | 22 | 5.55 | — | — | 14 | 8.71 | 8 | 15.3 |
| — | — | — | — | — | — | 25 | 1.20 | 24 | 1.25 |
| 56 | 1.66 | 42 | 2.21 | 32 | 2.91 | 29 | 3.21 | 21 | 4.43 |
| 16 | 2.75 | 11.5 | 3.83 | 10 | 4.40 | 8 | 5.50 | 4 | 11.00 |
| 43.5 | 2.35 | 37 | 2.76 | 29 | 3.52 | 25 | 4.08 | 17 | 6.00 |
| 57 | 1.28 | 58 | 1.26 | 61 | 1.20 | 57 | 1.28 | 59 | 1.24 |
| (247.5) | 1.14 | (242) | 1.17 | (254) | 1.11 | (236) | 1.20 | (229) | 1.24 |
| — | — | — | — | (230) | 1.03 | (219) | 1.08 | (221) | 1.07 |
| — | — | — | — | — | — | (382) | 1.11 | (361) | 1.18 |
| (127) | 1.06 | (131) | 1.02 | (120) | 1.12 | (123) | 1.09 | (112) | 1.20 |
| 63 | 1.19 | 52 | 1.44 | 40 | 1.88 | — | — | 27 | 2.78 |
| 20 | 1.20 | 16 | 1.50 | 14 | 1.72 | 12 | 2.00 | 11 | 2.18 |
| 13 | 2.62 | 10 | 3.4 | 5.5 | 6.2 | 4 | 8.5 | 3 | 11.3 |
| 34 | 1.62 | 35 | 1.57 | 42 | 1.31 | 35 | 1.57 | 33 | 1.67 |
| 262 | 1.26 | 224 | 1.48 | 188 | 1.76 | 155 | 2.14 | 130 | 2.55 |
| 79 | 1.39 | 60 | 1.84 | 42 | 2.62 | — | — | 29 | 3.80 |
| 33 | 1.15 | 35 | 1.09 | 34 | 1.12 | 32 | 1.19 | 32 | 1.19 |

active centre of the enzyme molecule that combines with TB and choline derivatives.

Regarding the action of choline on the esterase activity of *Helix* blood (Fig. 20), straight lines were also obtained by plotting v/v' against choline concentration. This was shown in the hydrolysis of ACh, ASaCh, and AAn, in which cases choline inhibited competitively. The inhibition in the AAn hydrolysis was much stronger than in the ACh- or ASaCh-hydrolysis which means that

AA_n has a lower affinity for the enzyme than ACh and ASaCh. These two esters seem to have about the same affinity for the *Helix*-blood ChE.

The hydrolysis of TB by *Helix* blood was also inhibited by choline, but non-competitively and less than the hydrolysis of choline esters. Two different mechanisms therefore are likely to act in the enzymic cleavages of TB and ACh respectively.

The esterase activity of the dart sac was also inhibited by choline (Table 32). The inhibition was stronger in the hydrolysis of ASaCh than in that of ACh. The enzyme affinity of ACh is therefore greater than that of ASaCh; further proofs for this statement are given in Chapter XII when the experiments with mixtures of substrates are reported. The action of choline in these cases is not likely to be competitive (see p. 138).

The hydrolysis of TB by the dart sac was inhibited very slightly and non-competitively. This was also the case for the tributyrinase activities of guinea-pig liver and cow liver (Table 32). As regards the liver esterase(s) of guinea pig, choline had a very weak action also on the hydrolysis of BzCh and ASaCh, and the inhibition was not competitive.

The results obtained with erythrocytes and brain are listed in Table 32 and shown graphically in Fig. 21. The ChE activity was strongly inhibited in both cases, but it was not expected that the same simple relationships would hold in these cases as those found for the ChE of horse plasma and *Helix* blood. Plotting v/v' against the concentration of choline straight lines were not obtained for the erythrocyte and brain enzymes, but S-shaped curves instead. The inhibition seems to be competitive, for all curves gave the intercept "one" by extrapolating to zero choline-concentration. Such were the results obtained irrespective of substrate studied (ACh, MeCh, ASaCh). It will be remembered that all results reported above have pointed to the identity of the erythrocyte and brain ChE. The curves in Fig. 21 provide further evidence of this identity.

The S-shaped curves would possibly indicate the formation of an active complex EIS, capable of breakdown. The rapid increase in inhibition by high choline concentrations might then be due to the formation of the complex EI₂. The relationship between degree of inhibition and inhibitor concentration in the hydrolysis of ACh, however, will be understood when the inhibition as function of substrate concentration has been discussed

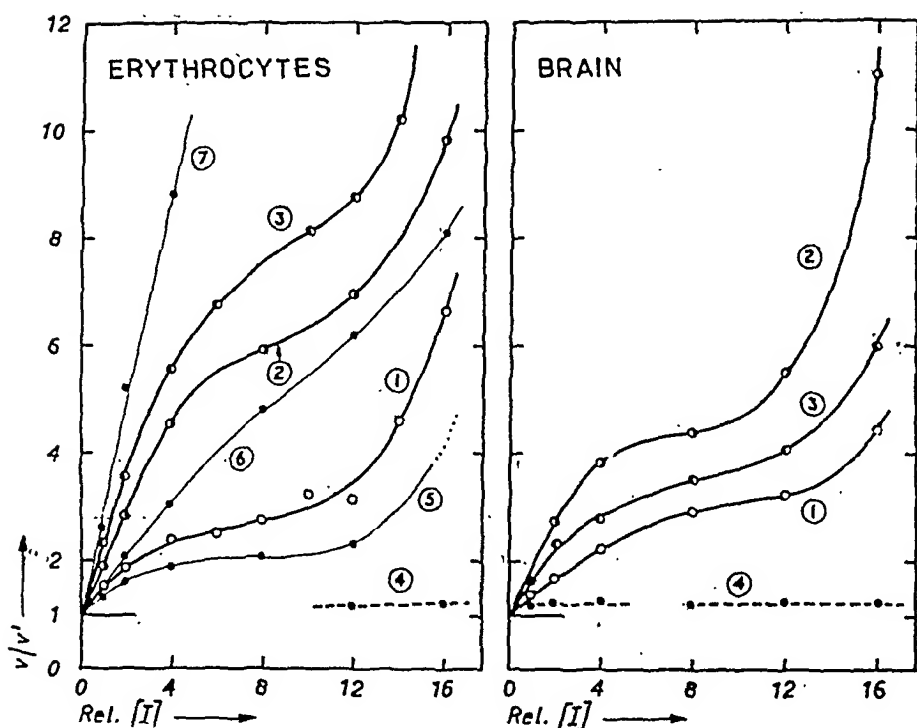


Fig. 21. Inhibition of ChE from cow erythrocytes and dog brain by choline. Data from Table 32.

1. ACh(3); 2. MeCh(3); 3. ASaCh(3); 4. TB(3); 5. ACh(2); 6. ACh(4); 7. ACh(5).

(see next Section, p. 135). It is therefore not necessary to suppose the formation of an active EIS complex in this case as an explanation of the results recorded in Fig. 21. In the hydrolysis of ASaCh the mechanism of inhibition by choline seems to be more complicated (p. 136).

The hydrolysis of TB by erythrocytes and brain was very slightly influenced by choline (Table 32 and Fig. 21).

C. INHIBITION AS FUNCTION OF SUBSTRATE CONCENTRATION

The inhibiting action of choline was also studied at various substrate concentrations. The results are found in Figs. 22—29. In some cases they were analysed by the LINEWEAVER-BURK procedure.

1. *Horse plasma*. The hydrolysis of ACh by horse plasma was strongly inhibited by choline (Fig. 22). As expected, pK'_s (in the presence of choline) was less than pK_s (in its absence), which means that greater ACh concentrations were needed for saturating

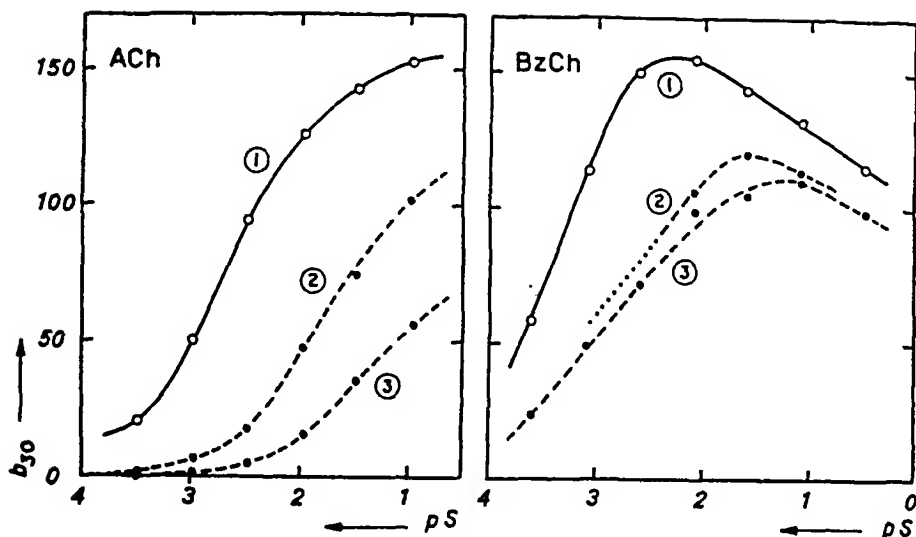


Fig. 22. Activity-pS curves for the enzymic hydrolysis of ACh and BzCh by horse plasma in the presence of added choline.

| Choline | ACh
(50 μ l. plasma) | | Choline | BzCh
(100 μ l. plasma) | | |
|--------------------------------|-----------------------------|---------|-----------------------------|-------------------------------|-------------------|--------|
| | V_{\max} | pK'_s | | V_{opt} | pS_{opt} | pS_1 |
| 1. — | 155 | 2.6 | 1. — | 156 | 2.2 | 3.4 |
| 2. 3.58×10^{-2} M... | 155 | 1.55 | 2. 7.16×10^{-2} M | 120 | 1.65 | (3.0) |
| 3. 10.74×10^{-2} M... | 155 | < 1 | 3. 10.74×10^{-2} M | 112 | 1.2 | 2.95 |

Cf. Fig. 23 for graphical analysis.

the enzyme. Straight lines were obtained when $1/[S]$ was plotted against $1/b_{30}$ (Fig. 23; cf. Equation 6); b_{30} expresses the reaction velocities v and v' when choline is absent and present respectively. The ordinate intercept ($1/V_{\max}$) is 6.5×10^{-3} which gives $V_{\max} = 154$ (155 is more correct, obtained by the procedure described on p. 99). The slopes of the lines multiplied with 155 give K_s and K'_s respectively. In two experiments with various choline concentrations, the dissociation constant K_I of the complex EI was found to be 3.5×10^{-3} and 3.2×10^{-3} respectively. Evaluating K_I from the data of Fig. 20, 3.2×10^{-3} was obtained. These results clearly indicate that ACh and choline compete for the same active centre of the enzyme molecule and that ACh has a somewhat higher affinity ($= 100$) for the enzyme than choline ($= 78$; according to ZIRFF *et al.*, 87).

It was shown in Chapter IX (Fig. 8) that the enzymic hydrolysis of BzCh is depressed by high substrate concentrations. This depressing effect is assumed to be due to the formation of complexes with more than one substrate molecule (ES_2 , ES_3 ...) (cf. LINE-

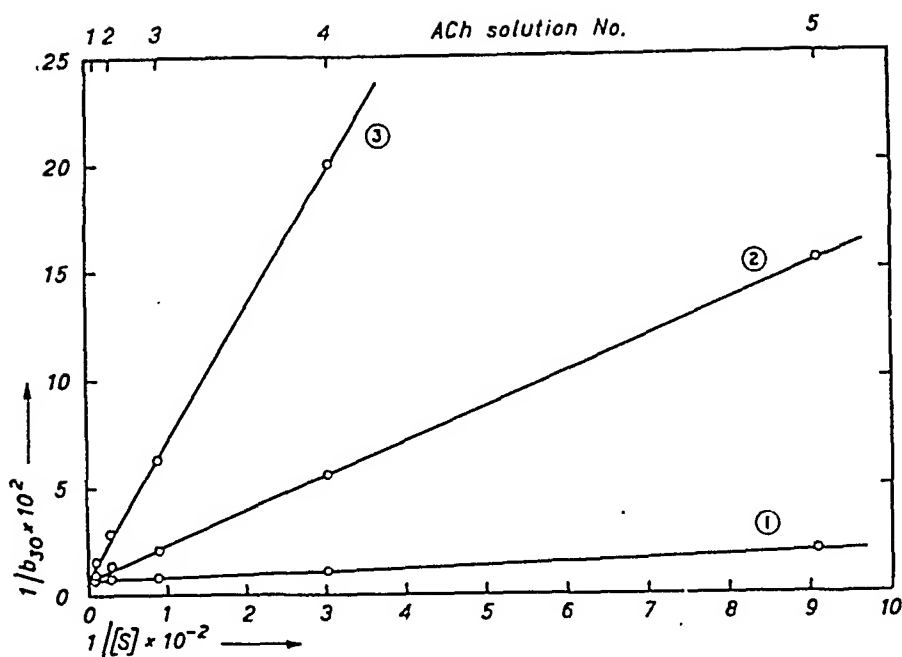


Fig. 23. Effect of choline on the enzymic hydrolysis of ACh by horse plasma. Results in Fig. 22 analysed by the LINEWEAVER-BURK procedure.

| [I] | K_S, K'_S resp. | $K_I = \frac{[I]}{K'_S/K_S - 1}$ |
|-----------------------------------|----------------------|----------------------------------|
| 1. — | 2.5×10^{-3} | — |
| 2. 3.58×10^{-3} M | 2.8×10^{-3} | 3.5×10^{-3} |
| 3. 10.74×10^{-4} M | 8.6×10^{-3} | 3.2×10^{-3} |
| | | 3.2×10^{-3} |

(according to Fig. 20)

WEAVER & BURK, 1934). Choline seems to be a competitive inhibitor also in the hydrolysis of BzCh, causing an increase of optimum substrate concentration and a decrease of optimum velocity (cf. below, cow erythrocytes).

2. *Cow erythrocytes*. As shown in the foregoing section choline also inhibits the ChE activity of erythrocytes (Fig. 24). In the presence of choline the optimum substrate concentration was changed to higher concentrations, the more choline added, the lower the value of pS_{opt} . In an ACh(3) solution (1.10×10^{-2} M) the substrate concentration is higher relatively to the optimum substrate concentration. When choline was added in increasing concentrations to such a solution, pS_{opt} approached that corresponding to 1.10×10^{-2} . In the presence of still higher choline concentrations, this substrate concentration was too low to give optimum enzyme activity. Consequently the enzyme activity increased with increasing substrate concentration ($> 1.10 \times 10^{-2}$)

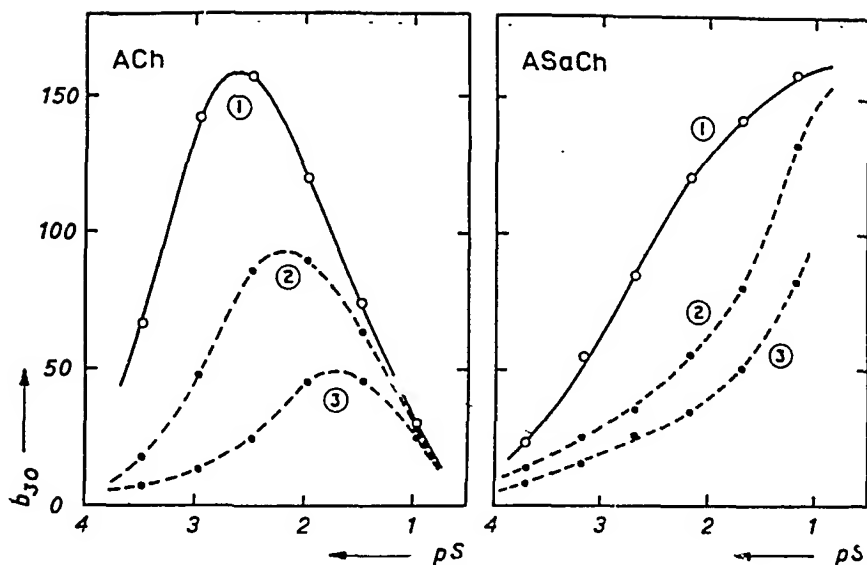


Fig. 24. Activity-pS curves for the enzymic hydrolysis of ACh and ASaCh by cow erythrocytes (100 μ l.) in the presence of added choline.

| Choline | | ACh | | | | Choline | | ASaCh | |
|---------|--------------------------|-----------|-----------|--------|--------|---------|-------------------------|-----------|--------------|
| | | V_{opt} | pS_{op} | pS_1 | pS_2 | | | V_{max} | $pK_S^{(c)}$ |
| 1. | — | 158 | 2.6 | 3.4 | 1.5 | 1. | — | 168 | 2.7 |
| 2. | 0.895×10^{-2} M | 94 | 2.2 | 3.0 | 1.25 | 2. | 1.79×10^{-2} M | ? | ? |
| 3. | 3.58×10^{-2} M | 50 | 1.7 | 2.5 | 1.0 | 3. | 3.58×10^{-2} M | ? | ? |

Cf. Fig. 25 for graphical analysis.

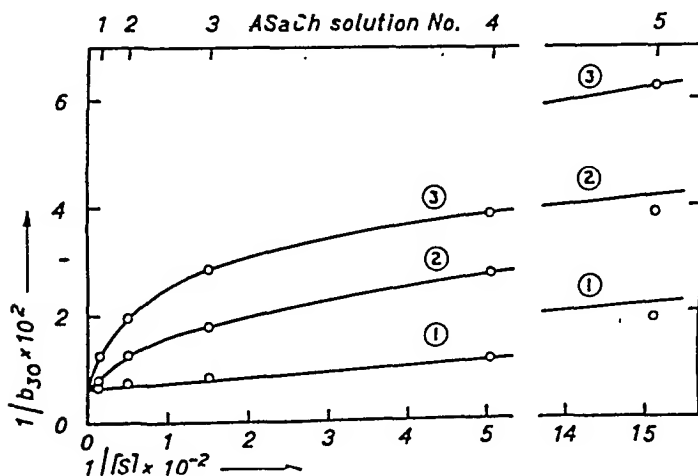


Fig. 25. Effect of choline on the enzymic hydrolysis of ASaCh by cow erythrocytes. Results in Fig. 24 analysed by the LINEWEAVER-BURK procedure.

1. No choline; 2. 1.79×10^{-2} -M choline; 3. 3.58×10^{-2} -M choline.

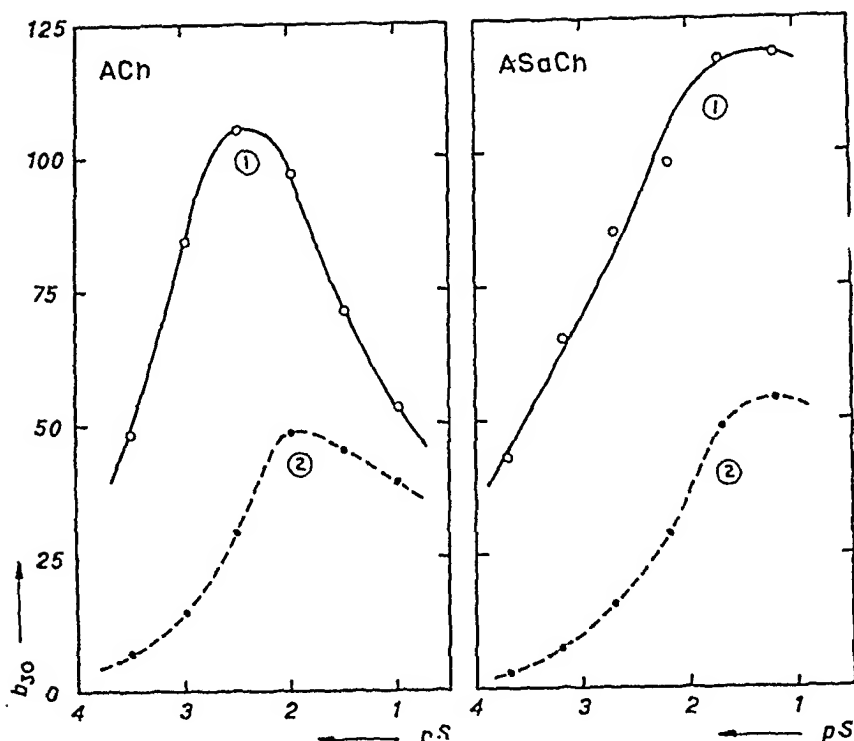


Fig. 26. Activity-pS curves for the enzymic hydrolysis of ACh and ASaCh by dog brain (100 mg.) in the presence of added choline.

| Choline | ACh | | | | ASaCh | |
|----------------------------|-----------|------------|--------|--------|-----------|--------------|
| | V_{opt} | pS_{opt} | pS_1 | pS_2 | V_{max} | $pK_S^{(c)}$ |
| 1. — | 105 | 2.4 | 3.4 | 1.0 | 119 | 3.25 |
| 2. $3.58 \times 10^{-2} M$ | 49 | 1.9 | 2.65 | ? | 54 | 2.25 |

when such a high concentration of choline was present, it decreased on the other hand in the absence of choline or in the presence of low choline concentrations. The degree of inhibition (v/v') therefore may not be expected to increase with increasing choline concentration according to Equation (7) or (11) (p. 104). A given substrate concentration above the optimum will tend to become optimal with increasing choline concentration. Consequently the enzyme activity comes nearer to its V_{opt} , reaching this at a given choline concentration. Therefore v/v' increases more slowly than expected. This explains most probably the results recorded in Fig. 21. When the substrate concentration is lower than the optimum one and the complex ES_2 not formed, the inhibition expressed by v/v' should be proportional to inhibitor concentration as is to be expected in a purely competitive inhibition. Such was the result in an experiment with the ACh solution No. 5 ($1.10 \times 10^{-3} M$) (Fig. 21).

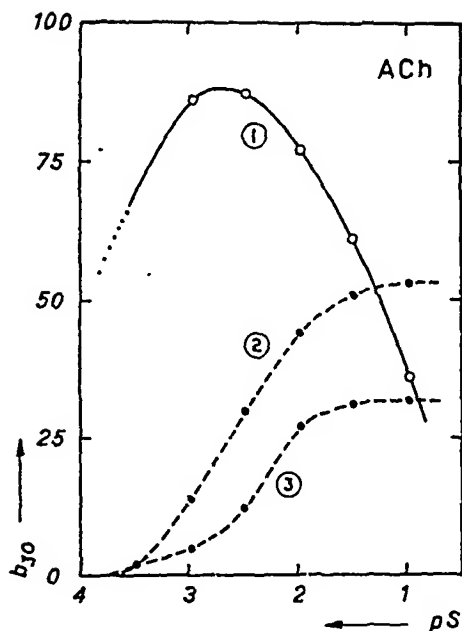


Fig. 27. Activity-pS curves for the enzymic hydrolysis of ACh by *Helix* blood (10 μ l.) in the presence of added choline.

| Choline | V_{opt} | $(V_{\text{max}} ?)$ | pS_{opt} | pS_1 | $(pK'_S ?)$ | pS_2 |
|-----------------------------------|------------------|----------------------|-------------------|-------------|-------------|--------|
| 1. — | 88 | | 2.7 | ≈ 4 | | 1.1 |
| 2. 3.58×10^{-3} M | | 53 | (≈ 1) | | 2.6 | — |
| 3. 10.74×10^{-3} M | | 32 | (≈ 1) | | 2.4 | — |

The hydrolysis of ASaCh gives a familiar dissociation curve, as shown in Fig. 24 (cf. Fig. 12). The action of choline on this reaction is more complicated than in other cases, also demonstrated in Fig. 25 where the results are analysed by the LINEWEAVER-BURK procedure. At low and high substrate concentrations respectively, the enzyme seems to act upon ASaCh in different ways. At high substrate concentrations the inhibition by choline was much less than expected in a competitive inhibition. At low and medium ASaCh concentrations the inhibition was considerable, choline presumably acting upon the same active centre that combines with ACh when this substrate is in low concentrations. Consequently ASaCh of low and medium concentrations is acted upon by the same active enzyme-centre that combines with ACh and choline. At high ASaCh concentrations another mechanism sets in, not competitively disturbed by choline. Further evidence for this assumption has been given above (p. 112).

3. *Dog brain*. As pointed out many times in this paper the ChE activity of brain is similar to the enzyme activity of erythrocytes. Also the action of choline on the two enzymes is the same (Fig. 26). The interpretation of this action discussed for the erythro-

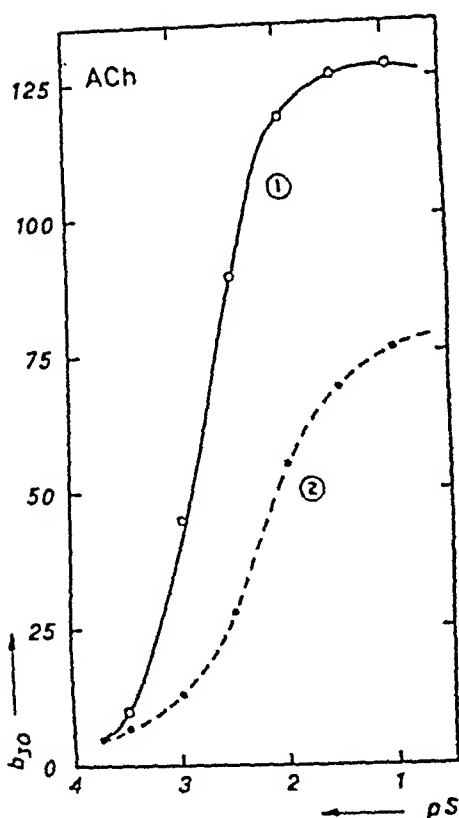


Fig. 28. Activity-pS curves for the enzymic hydrolysis of ACh by the dart sao (10 mg.) in the presence of added choline. Cf. Fig. 29 for graphical analysis.

| Choline | V_{\max} | $pK_S^{(1)}$ |
|-----------------------------------|------------|--------------|
| 1. — | 130 | 2.7 |
| 2. 10.74×10^{-3} M | 80 | 2.3 |

cyte ChE is most probably true also for the brain ChE. The inhibition of the ASaCh hydrolysis was not competitive which is in agreement with the results obtained in the experiments with erythrocytes.

4. *Helix* blood. The inhibition of the ChE activity of *Helix* blood by choline seems to be more complicated and is hardly capable of interpretation in the same way as the action of choline on the specific ChE of erythrocytes and brain. The activity was inhibited by excess of substrate, but the action of choline on the enzyme was not of the same kind as for the specific ChE. This is shown in Fig. 27. The inhibition seems to be competitive at low substrate concentrations. The action of choline was such that at high substrate concentrations and relatively low choline concentrations, choline had a weak activating effect on the enzyme activity. In the presence of choline, excess of substrate did not depress the activity. It will be remembered that the specific ChE (erythro-

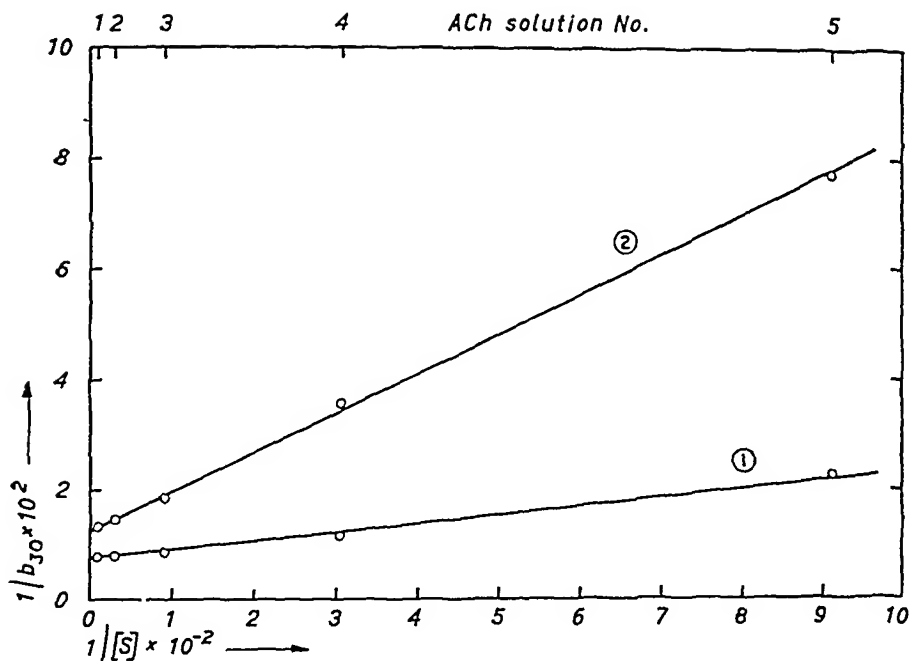


Fig. 29. Effect of choline on the enzymic hydrolysis of ACh by the dart sac. Results in Fig. 28 analysed by the LINEWEAVER-BURK procedure.

1. No choline; 2. 10.74×10^{-2} -M choline.

cytes, brain) and the *Helix*-blood ChE differ with respect to inhibition as function of choline concentration (Figs. 20 and 21).

5. *Dart sac*. The high ChE activity of the dart sac gave still another pattern (Fig. 28). Choline inhibited the enzyme, but the inhibition was not competitive. This is demonstrated in Fig. 29 where the results are analysed by the LINEWEAVER-BURK procedure. The ordinate intercept is higher in the presence of choline than in its absence, that is, V_{\max} is lower when choline has been added.

D. SUMMARY OF CHAPTER X

As expected choline exerted a competitive inhibition on ChE. But this was actually true only in some definite cases, demonstrated in experiments with varying choline concentrations as well as varying substrate concentrations.

The inhibition was purely competitive for the ChE activity of horse plasma, shown with ACh, BzCh, and ASaCh as substrates. Choline acted also competitively on the *Helix*-blood enzyme when it hydrolysed ACh, ASaCh, or AAn, and the concentrations of the substrates were not too high. At very high ACh concentrations

the *Helix*-blood ChE seems not to be acted upon by choline to give competitive inhibition. In the hydrolysis of BzCh by horse plasma, choline also exerted competitive inhibition at low substrate concentrations. Choline only slightly inhibited the TB hydrolysis by the enzymes of horse plasma and *Helix* blood respectively and in both cases the inhibition was non-competitive.

The ChE of erythrocytes and brain were also inhibited by choline. In plotting the degree of inhibition (expressed by v/v') against choline concentration, the same characteristic S-shaped curves were obtained in both cases, irrespective of substrate (ACh, MeCh, ASaCh). Choline inhibited competitively the hydrolysis of ACh. The S-shaped curves in this case have been interpreted on the basis that choline causes a shift of the optimum substrate concentration to higher concentrations. The inhibition of the two enzymes in the hydrolysis of ASaCh was more complicated and the enzyme mechanism seems not to be the same at high and low substrate concentrations. The hydrolysis of TB was inhibited very slightly and non-competitively.

The ChE activity of the dart sac was inhibited by choline, but the inhibition was not competitive.

CHAPTER XI

EFFECTS OF SOME FURTHER SUBSTANCES ON CHOLINESTERASE ACTIVITY

A. INTRODUCTION

Characteristic of the literature on the chemistry of ChE is the great variety of observations made on the effects of different compounds on the enzyme activity. A complete list of these substances has been given in Chapter II (Table 4). The purpose of these studies, in most cases, have been to find a possible explanation of certain physiological and pharmacological and also clinical events in terms of a reduction or potentiation in the ChE activity. Such a correlation has also been tried in a great variety of pathological states, as reported in Chapter I (p. 14). But in very few cases conclusions as to the relation between ChE activity and diseases or drug functions could be drawn. The pharmacological actions of physostigmine, prostigmine, and similar drugs,

TABLE 33. *Inhibition of ChE from*
Substrate solutions No. 3.

| Enzyme | | Sub-
strate | No in-
hibitor
present | Inhibitor | | | |
|------------------------------|---------------------------------|----------------|------------------------------|--|------|---|------|
| Preparation | μ l. mg.
per
2.00 ml. | | | Physostigmine
3.63×10^{-6} M | | Methylene blue
4.01×10^{-4} M | |
| | | | | b_{30} | inh. | b_{30} | inh. |
| Purified horse-
serum ChE | C_f
$\approx 3\ 000$ | ACh | 153 | 2 | 99 | 1 | 100 |
| | | BzCh | 68 | 9.5 | 86 | 3.5 | 95 |
| | | ASaCh | 128 | 16 | 87 | 8 | 94 |
| | | TB | 42 | 7 | 83 | 5 | 88 |
| <i>Scyllium</i> plasma | 200 | ACh | 18.5 | 0 | 100 | 0 | 100 |
| | 200 | MeCh | 10 | 2 | 80 | — | — |
| | 200 | ASaCh | 27 | 0 | 100 | 2 | 93 |
| Cow erythrocytes | 50 | ACh | 93 | 12 | 87 | 5 | 95 |
| | 50 | MeCh | 57 | 11 | 81 | 7 | 88 |
| | 50 | ASaCh | 105 | 15 | 86 | 17 | 84 |
| | 50 | TB | 25 | 25 | 0 | 25 | 0 |
| Dog brain | 50 | ACh | 35 | 8 | 77 | 0 | 100 |
| | 50 | MeCh | 30 | 4 | 87 | 5 | 83 |
| | 50 | ASaCh | 48 | 6 | 87 | 6 | 87 |
| | 50 | TB | 36 | 32 | 11 | 25.5 | 29 |
| Guinea-pig liver | 100 | ACh | 13 | 5 | 62 | 2 | 85 |
| | 50 | BzCh | 230 | 191 | 17 | 234 | 0 |
| | 50 | ASaCh | 225 | 178 | 21 | 215 | 4 |
| | 25 | TB | (360) | (355) | 0 | (360) | 0 |
| <i>Sepia</i> "liver" | 50 | ACh | 82 | 8 | 90 | 4 | 95 |
| | 50 | ASaCh | 38 | 24 | 37 | 20 | 48 |
| | 50 | TB | 54 | 43 | 20 | 38.5 | 29 |
| <i>Helix</i> blood | 20 | ACh | 107 | 8 | 93 | 4 | 96 |
| | 20 | MeCh | 43 | 5 | 88 | 9 | 79 |
| | 20 | ASaCh | 35 | 5.5 | 84 | 3.5 | 90 |
| | 20 | AAn | 47 | 2 | 96 | 3 | 94 |
| | 200 | TB | 49.5 | 29 | 41 | — | — |
| Dart sac | 25 | ACh | 308 | 6 | 98 | 50 | 84 |
| | 25 | MeCh | 23 | 0 | 100 | — | — |
| | 25 | ASaCh | 118 | 10 | 92 | 15 | 87 |
| | 25 | TB | 31 | 21 | 32 | — | — |

and also those of di-isopropyl fluorophosphate, however, are exceptions and chiefly accounted for on the inhibition of the ChE activity. These substances are also known as the most powerful "anticholinesterases" (see p. 28). Some recent papers deal with the actions of various substances in order to find out possible criteria for a differentiation of different choline-ester splitting enzymes. In the light of recent results about the specificity of

Various Sources by Different Substances

Inhibition expressed in per cent.

present

| Caffeine
7.07×10^{-4} M | | Quinine HCl
3.78×10^{-4} M | | Neurine Br
9.03×10^{-4} M | | Cystine
6.24×10^{-4} M | | Clupeine
0.05 % | | Gum arabic
0.05 % | |
|-------------------------------------|------|--|------|---------------------------------------|------|------------------------------------|------|--------------------|------|----------------------|------|
| b_{30} | inh. | b_{30} | inh. | b_{30} | inh. | b_{30} | inh. | b_{30} | inh. | b_{30} | inh. |
| 151 | 1 | 20 | 87 | 142 | 7 | 173 | + | 27 | 82 | 185 | + |
| 66.5 | 2 | 48 | 29 | 68.5 | 0 | 74 | + | 37.5 | 45 | 74.5 | + |
| 129 | 0 | 54 | 58 | 128 | 0 | 138 | + | 48 | 62 | 153 | + |
| 42 | 0 | 7.5 | 82 | 39 | 7 | 48 | + | 22 | 48 | 53.5 | + |
| 15 | 19 | 4 | 78 | — | — | — | — | — | — | — | — |
| — | — | — | — | — | — | — | — | — | — | — | — |
| — | — | — | — | — | — | — | — | — | — | — | — |
| 74 | 20 | 82 | 12 | 83 | 11 | 90 | 3 | 113 | + | 86 | 8 |
| 48 | 16 | 49 | 14 | 46 | 19 | 59 | 0 | 38 | 33 | 59 | 0 |
| 103 | 2 | 88 | 16 | 85 | 19 | 105 | 0 | 64 | 39 | 84 | 20 |
| 26 | 0 | 18 | 28 | 26 | 0 | 19.5 | 22 | 22.5 | 10 | 25 | 0 |
| 27.5 | 21 | 31 | 11 | 30 | 14 | 36.5 | 0 | 38 | (0) | 43 | + |
| 23.5 | 21 | 24 | 20 | 25 | 17 | — | — | 16 | 47 | 32 | 0 |
| — | — | 36 | 25 | — | — | — | — | — | — | — | — |
| — | — | 36 | 0 | — | — | — | — | — | — | — | — |
| 10 | 23 | 2 | 85 | 10 | 23 | 10 | 23 | 11 | 15 | — | — |
| 232 | 0 | 26 | 89 | 226 | 2 | 233 | 0 | 293 | + | 218 | 5 |
| 224 | 0 | 85 | 62 | 222 | 1 | 197 | 12 | 253 | + | 260 | + |
| (355) | 0 | (325) | 10 | — | — | — | — | (360) | 0 | (400) | + |
| 82 | 0 | 23 | 72 | — | — | — | — | 82 | 0 | 80 | 2 |
| 38 | 0 | 30 | 21 | — | — | — | — | 38.5 | 0 | 38 | 0 |
| — | — | 50 | 7 | — | — | — | — | — | — | — | — |
| 104 | 3 | 126 | + | 85 | 20 | 128 | + | 107 | 0 | 132 | + |
| — | — | — | — | — | — | — | — | 20 | 53 | — | — |
| 21 | 40 | 33 | 6 | 25 | 28 | — | — | 17 | 51 | — | — |
| 38 | 19 | 32 | 32 | 34 | 28 | — | — | 20 | 57 | — | — |
| — | — | — | — | — | — | — | — | — | — | — | — |
| 299 | 3 | 133 | 57 | 295 | 4 | — | — | 313 | 0 | — | — |
| — | — | — | — | — | — | — | — | — | — | — | — |
| 119 | 0 | 39 | 67 | 119 | 0 | — | — | 70 | 41 | — | — |
| — | — | — | — | — | — | — | — | — | — | — | — |

ChE, the present author has studied the effects of some substances on the esterase activities of various sources. Strong evidence of the identity of two enzymes may be the fact that both are affected in the same way by a certain compound.

One of the most characteristic inhibitors of ChE is physostigmine, the action of which has been discussed in certain cases in Chapter IX. Also the characteristic effect of clupeine has been

reported there. For comparison the actions of these agents are also listed in Table 33 which in addition summarises the results obtained with other substances, the effects of which will be considered next. The experiments have been carried out with the substrate solutions No. 3. In the side bulb of the flask, 0.20 ml. of the enzyme preparation was mixed with 0.20 ml. of the inhibitor solution. The concentrations of the inhibitors are found in Tables 7 and 33.

B. RESULTS

1. *Physostigmine salicylate*. Previous investigations on the inhibiting action of physostigmine on ChE are reviewed in Chapter II (p. 29). The inhibition is said to be competitive. As shown in Chapter IX, physostigmine salicylate (3.63×10^{-6} M) inhibits the ChE activity at all substrate concentrations completely or almost completely. Complementary results are found in Table 33. The hydrolysis of TB by blood serum was strongly inhibited, but not that by erythrocytes and brain respectively. The tributyrinase activities of *Sepia* "liver" and *Helix* blood were inhibited but not strongly; physostigmine did not affect that activity of other liver preparations.

An exception to the general rule already noted, that the enzymic destruction of all choline esters are completely retarded by small concentrations of physostigmine, is the effect of this inhibitor on the rapid breakdown of BzCh by guinea-pig liver which was only slightly influenced (17 per cent). The hydrolysis of ACh, however, was inhibited to about 60 per cent. This agrees with an assumption that two distinct esterases exist in guinea-pig liver.

Physostigmine inhibited in the concentration of 3.63×10^{-6} M the hydrolysis of ASaCh by guinea-pig liver to about 20 per cent. Fig. 30 shows the hydrolysis of this ester with and without inhibitor. The two curves approach each other slowly and they run together when complete destruction of the choline-ester linkage is reached, that is, the reaction which is supposed to be inhibited by physostigmine. The breakdown of the acetyl-salicylic linkage, most probably, is not influenced by physostigmine, for the hydrolysis of ASa, proceeding at a very high rate through the action of guinea-pig liver, was not inhibited by this drug (t_{30} , in experiments with and without inhibitor, was 184 and 187 respectively).

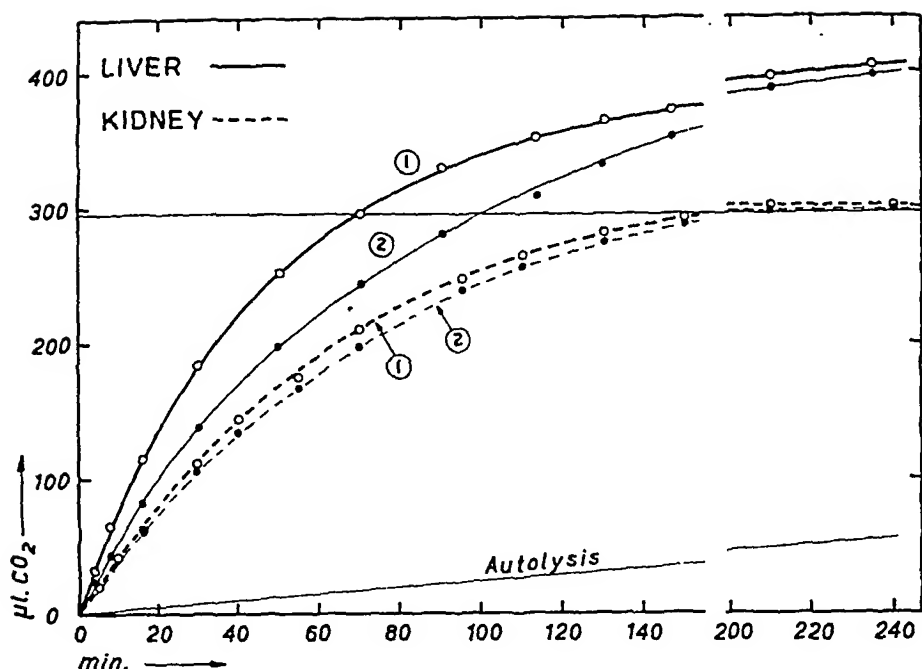


Fig. 30. Total (enzymic + non-enzymic) hydrolysis of ASaCh by guinea-pig liver (50 mg.) and cow kidney (100 mg.) respectively. Action of physostigmine on these reactions. "Autolysis" refers to non-enzymic hydrolysis.

1. No inhibitor; 2. 3.63×10^{-6} -M physostigmine salicylate.

Fig. 30 shows also the results obtained in similar experiments performed with cow kidney which splits ASaCh at a high rate, but BzCh at a low rate. This reaction was practically un-influenced by physostigmine. It is therefore most probably the acetyl-salicylic linkage only that is destroyed and this reaction is *a priori* not inhibited by physostigmine. As is supposed above (p. 119), cow kidney probably contains a "salicyl-esterase" with other properties than a ChE. Similar results have been obtained with guinea-pig kidney and also with *Spirographis* blood (AUGUSTINSON, 1947).

2. *Methylene blue* is a strong inhibitor of serum ChE, first shown by RENTZ (1940) and by MASSART and DUFAYT (1940 c, 1941 a, b). This action is due to the presence of the quaternary ammonium ion, for the leuco form of the dye has no inhibiting effect. The anti-ChE activity of methylene blue and other oxidation-reduction systems has been discussed in detail by KLEIN (1944) and by TORDA and WOLFF (1944), using blood serum as an enzyme preparation.

Table 33 shows that methylene blue in 4.10×10^{-4} -M solutions inhibits the enzymic hydrolysis of choline esters as well as TB

by horse serum to about the same high extent. This provides further evidence that the same enzyme splits ACh and TB. Potent inhibiting action was found on the ChE activity of shark plasma. The erythrocyte ChE was also strongly inhibited, but the hydrolysis of TB by the cells was not affected. This is in accordance with the assumption that two distinct enzymes are responsible for the hydrolysis of choline esters and TB. Similar results were obtained with brain which, in addition to a specific ChE, contains a tributyrinase, only slightly acted upon by methylene blue.

The low rate of the ACh hydrolysis by guinea-pig liver was strongly inhibited by the dye, but this was not the case in the BzCh and ASaCh reactions. It may therefore be supposed that this tissue contains an ACh-splitting enzyme in very low concentration and in addition a second esterase acting upon BzCh and ASaCh. The hydrolysis of TB was also unaffected by methylene blue. The *Labrus*-liver ChE was inhibited to about 76 per cent (b_{30} 108 and 28 respectively, substrate ACh). The inhibition of the *Sepia*-“liver” ChE was strong, that of its tributyrinase activity much weaker. The hydrolysis of ASaCh was inhibited to about 50 per cent. It is supposed that ASaCh is acted upon by two esterases in *Sepia* “liver”, one of which is strongly inhibited by methylene blue.

The ChE activity of *Helix* blood showed the same characteristic and strong inhibition by methylene blue; also the hydrolysis of AAn was inhibited almost completely. The dart-sac enzyme behaved in the same way.

3. *Caffeine* is said to inhibit the specific ChE selectively, shown for the first time by ZELLER and BISSEGER (1943) and later on confirmed by NACHMANSOHN and SCHNEEMANN (1945). In the experiments performed by the present author, the caffeine concentration was 7.07×10^{-4} M. It is clearly demonstrated in Table 33 that this compound does not inhibit the ChE activities of horse serum, *Sepia* “liver”, and the dart sac. On the other hand, the enzymic hydrolysis of ACh was inhibited by caffeine using erythrocytes, brain, and guinea-pig liver. The inhibition in all these cases was about 20 per cent. These findings offer further proofs that caffeine acts exclusively on the specific ChE, that type of choline-ester splitting enzymes supposed to exist in those tissues where caffeine acts as an anti-ChE. The enzyme of shark plasma was also inhibited by caffeine. This was expected from other findings indicating this plasma to contain a specific

ChE in low concentration. The tributyrinase activity was not inhibited by caffeine in any case.

The inhibition of the ChE activities of erythrocytes and brain as function of caffeine concentration is found in Fig. 31. The curves have the same characteristic S-shape as was found in the inhibition of these activities by choline (Fig. 21). The potency of caffeine to inhibit the specific ChE is not very great, but the distinguishing feature of the inhibition is undisputable. Caffeine is a well-known stimulant of the brain and the inhibition of the brain ChE by this drug is interesting from this point of view. The fact that the degree of inhibition is constant for a wide range of drug concentrations may be important in the prescription for using the drug therapeutically. If the relationship between the inhibition of the specific ChE and the drug concentration is also true for other "anticholinesterases" of high potency, *e.g.*, physostigmine, prostigmine, DFP, it will become of general value in the therapeutics of such compounds.

The hydrolysis of ACh by *Helix* blood, which in certain cases gives the same pattern as the hydrolysis catalysed by the specific ChE (erythrocytes, brain), was not influenced by caffeine (Table 33 and Fig. 31).

4. *Quinine hydrochloride*. Previous investigations have shown that quinine inhibits serum ChE, but has a weaker effect on the specific ChE of brain (for references, see Table 4). This has been confirmed by the studies performed here. Quinine hydrochloride (3.78×10^{-4} M) inhibited strongly the hydrolysis of choline esters and TB catalysed by serum, liver preparations, and the dart sac. The affinity of this drug for the specific ChE of red blood cells and brain was less.

A more detailed account on the action of quinine is found in Fig. 31, where the degree of enzyme inhibition (expressed by v/v') has been plotted against the concentration of inhibitor. The inhibition is not purely competitive, neither in the case of horse plasma nor of the dart sac. The activating effect on the hydrolysis of ACh by *Helix* blood is remarkable and not found in any other case.

5. *Neurine bromide*. The action of neurine $[(CH_3)_3N-CH=CH_2]^+$, the structure of which is close to that of choline, has not been considered previously in ChE studies. In 9.03×10^{-4} -M solution neurine bromide inhibited the ChE activities of erythrocytes, brain, and *Helix* blood relatively stronger (about 20 per

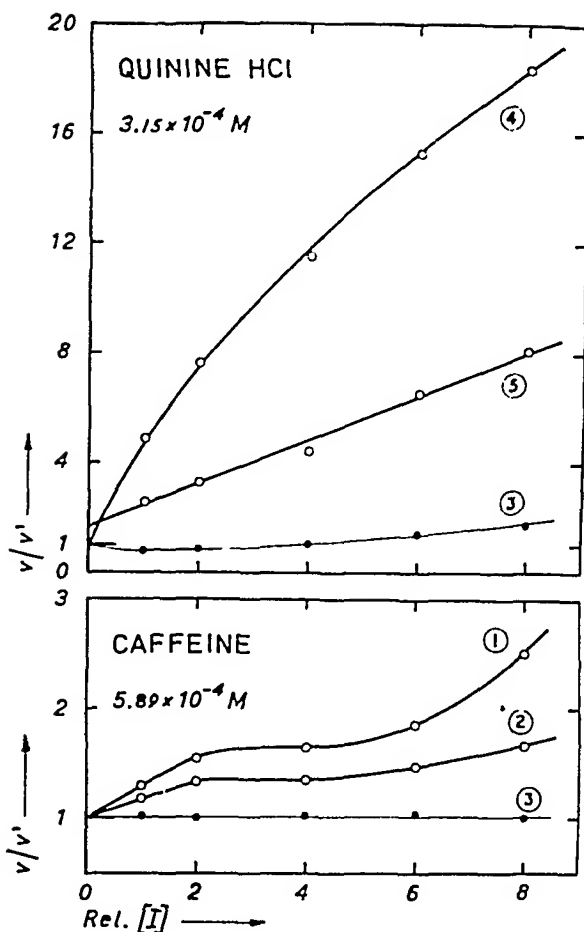


Fig. 31. Inhibition of ChE by caffeine and quinine hydrochloride respectively as function of inhibitor concentration. Absolute molar concentrations noted correspond to relative concentration "1". Substrate ACh(3).

Enzyme source $\left(= \frac{v}{b_{30}} \right)$

1. Cow erythrocytes (100 μ l.) 120
2. Dog brain (100 mg.) . 79
3. *Helix* blood (50 μ l.) .. 349
4. Horse plasma (25 μ l.) 92
5. Dart sac (25 mg.) 274

cent) than the activities of blood serum and the dart sac. Thus neurine behaved in the same way as caffeine. The low rate of the ACh hydrolysis catalysed by guinea-pig liver was also slowed down, but this was not the case in the BzCh and ASaCh reactions.

6. *Cystine*. As pointed out in Chapter II, NACHMANSON and LEDERER (1939 a, b) have supposed that the action of ChE (from electric organ) depends upon free SH groups. They found that oxidised glutathione, like cystine a disulphide, inhibits the enzyme activity. An inhibition of the ChE in leech extracts by cystine was observed by RIECHERT and SCHMID (1942). In contradistinction to these findings, STADIE, RIGGS and HAUGAARD (1945) have shown ChE to be very resistant to oxidising agents. In experiments performed by the present author it has been stated that cystine has a very weak or no effect on the ChE activities of various sources. The results, however, are not very consistent. Thus a small activation was observed with the non-

specific ChE of horse serum and also with the *Helix*-blood ChE. In other cases no or very inconsiderable inhibition was observed.

When the experimental part of this work was completed, WELS and REPKE (1947) announced their results from an investigation on the effects of certain thiol reagents on ChE. Confirming the author's observation they found cystine to have no inhibiting action on serum ChE (cat).

7. *Clupeine*. The action of clupeine on ChE was discussed in detail in Chapter IX where the characteristic change of pK_s and pS_{opt} in the presence of this protamine was demonstrated. This change of pS_{opt} for the specific ChE explains the fact that, in the case of ACh hydrolysis in solution No. 3, clupeine has no effect on the enzymes of erythrocytes, brain and *Helix* blood (Table 33). When other choline esters, however, were used, inhibition was observed, due to the non-alteration of pS_{opt} . The general effect of clupeine on ChE, the specific as well as the non-specific enzyme, is undisputable, however, and shown for certain. In sharp contrast to this pattern stands the activation of the guinea-pig liver esterase in the hydrolysis of BzCh and ASaCh (Table 33).

8. *Gum arabic* has a weak potentiating effect on the ChE activity in certain cases, shown in Chapter IX and Table 33. This action seems to be characteristic of the specific as well as the non-specific ChE.

C. SUMMARY OF CHAPTER XI

Physostigmine and methylene blue inhibited the ChE activity strongly in all cases, the specific ChE as well as the non-specific esterase. With a number of other compounds significant differences in affinity for various types of esterases were found. Caffeine and neurine inhibited the activity of the specific ChE almost exclusively. Quinine, on the other hand, showed strong inhibition of the non-specific ChE, the effect on the specific enzyme was much weaker. An acceleration of the ACh hydrolysis by *Helix* blood was observed in the presence of low quinine concentrations. The inhibiting effect of quinine was not competitive. Plotting the caffeine concentration against the degree of inhibition by this drug, S-shaped curves were obtained of the same kind as was found in experiments with choline. Cystine had a very weak or no effect on ChE.

According to these findings the same specific ChE occurs in erythrocytes and brain. The enzymes of *Helix* blood and the dart sac behave differently and are not identical with the esterases of horse serum or *Sepia* "liver".

CHAPTER XII

HYDROLYSIS OF MIXTURES OF SUBSTRATES

A. INTRODUCTION

In the foregoing Chapters a number of ways have been discussed which may be employed in establishing the identity of enzymes acting on several substrates. A further criterion of specificity has been obtained from measuring the rate of hydrolysis when an enzyme preparation acted upon a mixture of two substrates, and comparing the amount of CO_2 evolved with the amounts when the substrates were hydrolysed separately.

An enzyme preparation, which was found to catalyse the hydrolysis of two substrates, was allowed to act upon a mixture of these substrates. The rate of this reaction might be higher, equal to, or less than the rate of hydrolysis of that substrate alone which was split at the highest rate.

(1) If two separate esterases act upon each of two substrates, the amount split at any time in the mixture of the substrates should equal the sum of the amounts split at the same time when the two esters are hydrolysed separately, provided that the substrate concentrations are high enough to saturate the enzymes. In such a case the two reactions go on, each entirely independently of the other. This is the experimental results in the hydrolysis of ACh and TB by red blood cells (Fig. 32), brain (Fig. 33), *Labrus* liver (Fig. 34), and *Sepia* "liver" (Fig. 35), respectively.

The effect by mixing two substrates, however, must not be absolutely additive. Suppose, for instance, that one of the substrates (or its reaction products) inhibits the action of the other. In such a case the hydrolysis at any given time will be less in the mixture than the sum of the separate hydrolysis. This fact explains possibly the experimental results in some cases in the hydrolysis of ACh and TB (Figs. 33, 34).

(2) If two substrates are hydrolysed by the same enzyme, an additive effect will not be the result in a mixture of the substrates, except in very low substrate concentrations.¹ In the case of high substrate concentrations, the rate of hydrolysis should not be altered when a substrate with high affinity for the enzyme is mixed with a second substrate, the affinity of which is less. This is the experimental results in most reactions of mixtures of choline esters (Figs. 32—36). The fact is that ACh, in most cases, has the highest affinity for ChE, but this is not always true as will be seen next.

(3) In some cases a decrease of the rate of hydrolysis was observed when two substrates (choline esters) were mixed. This is the experimental results in the hydrolysis of ACh and AAn by *Sepia* "liver" (Fig. 35), and of ACh and ASaCh by *Helix* blood (Fig. 36). It is assumed that the enzyme affinity of the second substrate (AAn and ASaCh respectively) is the same or greater, and the separate rate of hydrolysis less than the corresponding data of the first substrate (ACh, in this case); hence, the result will be a decrease in the rate of hydrolysis in the mixture of the two substrates.

In the experiments of this kind, the main compartment of the flask was filled with 0.80 ml. of a substrate solution No. 3 and 0.80 ml. distilled water or 0.80 ml. of another substrate solution No. 3. As usual, the enzyme activity was expressed by b_{30} .

B. RESULTS

1. *Plasma*. It has been shown in the preceding Chapters that the blood plasma of various vertebrates most probably contain an enzyme which brings about the hydrolysis of choline esters and tributyrin as well. The results obtained in experiments with mixtures of substrates provide further evidence that human, horse, and guinea-pig plasma do not contain a specific ChE. Allowing the enzyme to act upon a mixture of ACh and TB, the reaction velocity was almost the same as when ACh alone was present at the same concentration. At this concentration, 5.5×10^{-3} M, the enzyme is not saturated with ACh, but this makes no difference in this case as the velocity is not altered by 3.3×10^{-3} -M TB.

¹ In a personal communication, Dr. V. P. WHITTAKER has pointed out that an additive effect will only be obtained when each substrate concentration is much smaller than the corresponding K_s .

The results are demonstrated by the data obtained with guinea-pig plasma (Fig. 32). Nor did the rate of ACh hydrolysis change when this substrate was mixed with 4.11×10^{-3} -M BzCh, thus indicating that the same enzyme catalyses the hydrolysis of ACh and BzCh.

2. *Erythrocytes*. In contrast to the plasma, the red blood cells contain two esterases at least, one splitting only choline esters and one splitting TB. At 5.5×10^{-3} -M ACh, optimum conditions are prevailing for enzymic hydrolysis in most cases by erythrocyte ChE. When TB was present at the same time a purely additive effect was observed, and the two substrates therefore were split each independently of the other. This is very well demonstrated in Fig. 32; the shaded area will explain the additive effect. In this case of guinea-pig erythrocytes, the value of b_{30} (283) agrees very well with the expected value ($184 + 100$) for a purely additive effect.

3. *Brain* (dog) also contains two enzymes acting upon choline esters and TB respectively. The additive effect in the mixture of ACh and TB, as shown in Fig. 33, was not pure, but indicated indisputably that the two substrates were destroyed by distinct esterases. All choline esters used in this series of experiments were split by the same specific ChE. The determinations were carried out at optimum substrate concentrations.

4. *Labrus liver*. This bony-fish liver contains a "specific" ChE which in addition brings about the hydrolysis of AAn (Fig. 34). The rate of hydrolysis in the mixture of ACh and ASaCh was somewhat lower than that of ACh alone, but higher than that of ASaCh alone. The fact that b_{30} for the hydrolysis of the mixture, 120, was about the mean of the hydrolysis of the substrates separately, or $\frac{1}{2}(148 + 88) = 118$, goes to prove that the affinities of ACh and ASaCh respectively for the liver enzyme are the same. The additive effect in the mixture of ACh and TB indicated that in addition to the ChE this tissue contains an esterase splitting TB.

5. *Sepia "liver"*. Two esterases are also present in the digestive gland ("liver") of cuttlefish. One catalyses the hydrolysis of ACh, MeCh, ASaCh, and AAn. This is demonstrated in Fig. 35. AAn seems to have a high affinity for the enzyme, but is split at a low rate. Consequently, the rate of ACh hydrolysis was decreased by adding AAn; both these esters compete for the same active group(s) of the enzyme molecule. It will be observed that the mean of the rates of the separate reactions was 60.5, very near the value

Fig. 33.

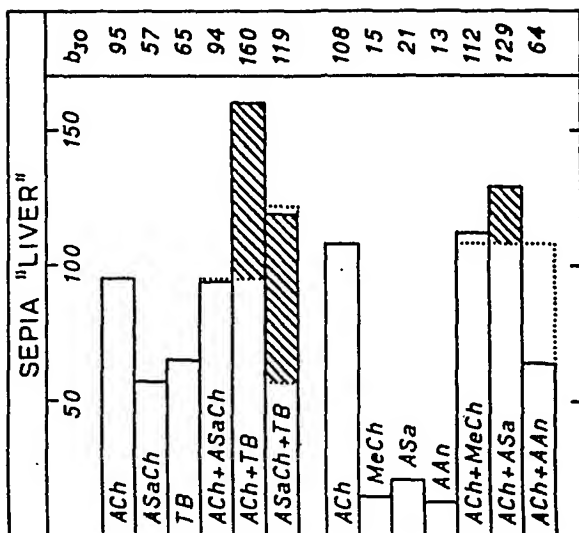
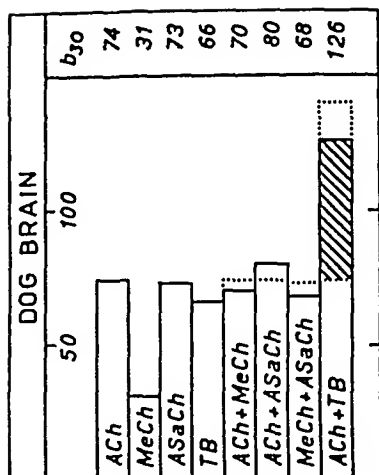


Fig. 35.

Fig. 32.

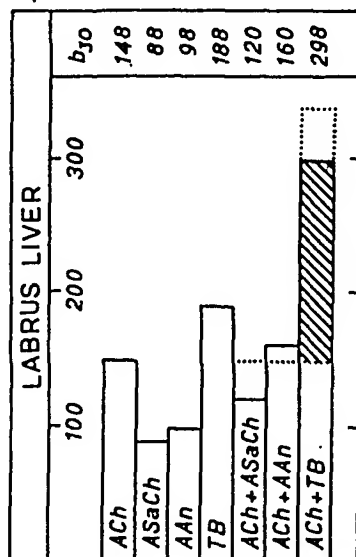
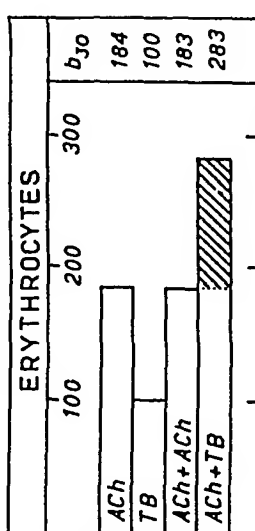
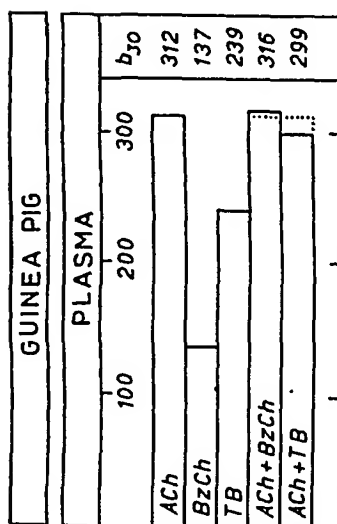


Fig. 34.

obtained in the mixture, 64. This seems to prove that the two substrates have about the same affinity for the enzyme. The hydrolysis of TB proceeded entirely independently of the destruction of ACh and ASaCh respectively, thus indicating that another esterase acts on the triglyceride. Also ASa is split by an enzyme not identical with ChE.

6. *Helix blood*. The blood of the edible snail seems to contain an enzyme which splits ACh at a very high rate and other choline esters at low rates, in addition also AAn. ASaCh reduced the hydrolysis of ACh to a very great extent which means that the affinity of ASaCh for the enzyme is greater than that of ACh. This is very clearly demonstrated in Figs. 36 and 37.

Fig. 37 shows the results of an experiment in which the reactions were allowed to proceed for a long time to give complete destruction of the substrates. Under the experimental conditions, the breakdown of ACh was ended in about 40 minutes when the amount of CO_2 evolved was equal to the calculated amount for complete destruction. In the presence of MeCh at the same time, the hydrolysis of this substrate started when ACh was completely used up, and went on more slowly than in the absence of ACh; this might be due to the inhibiting action of choline, formed in the hydrolysis of ACh. The relatively high affinity of ACh for the enzyme compared with that of MeCh explains the fact that MeCh was acted upon, only when ACh was completely destroyed. On the other hand, ACh and ASaCh in mixture were split simultaneously and at a rate which was between those of the two separate reactions. Both ester linkages of ASaCh are split, for the reaction went on after the calculated values for complete destruction of ACh and one of the linkages in ASaCh were reached. Then the hydrolysis approached slowly the theoretical value for the breakdown of both linkages.

7. *Dart sac*. Similar experiments have been performed with the dart sac. This tissue most probably contains one esterase, a ChE that hydrolyses ACh at a very high rate. It also destroys ASaCh at high rate and MeCh at low rate. Most probably the same enzyme acts upon TB; in mixtures of ACh and TB no additive effect was observed.

ACh was rapidly and completely split in an experiment, the results of which are shown in Fig. 38. In mixtures of ACh with MeCh and ASaCh respectively, the enzymic hydrolysis of the added ester started when ACh was almost completely used up.

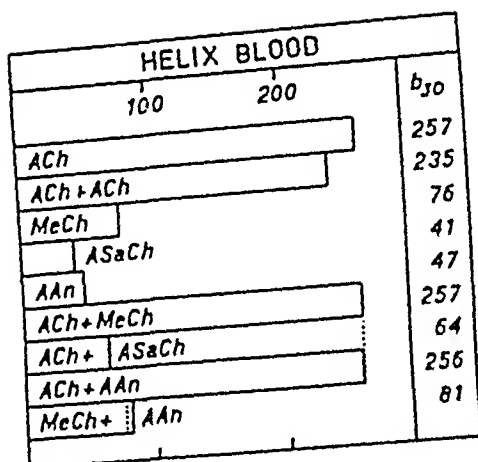
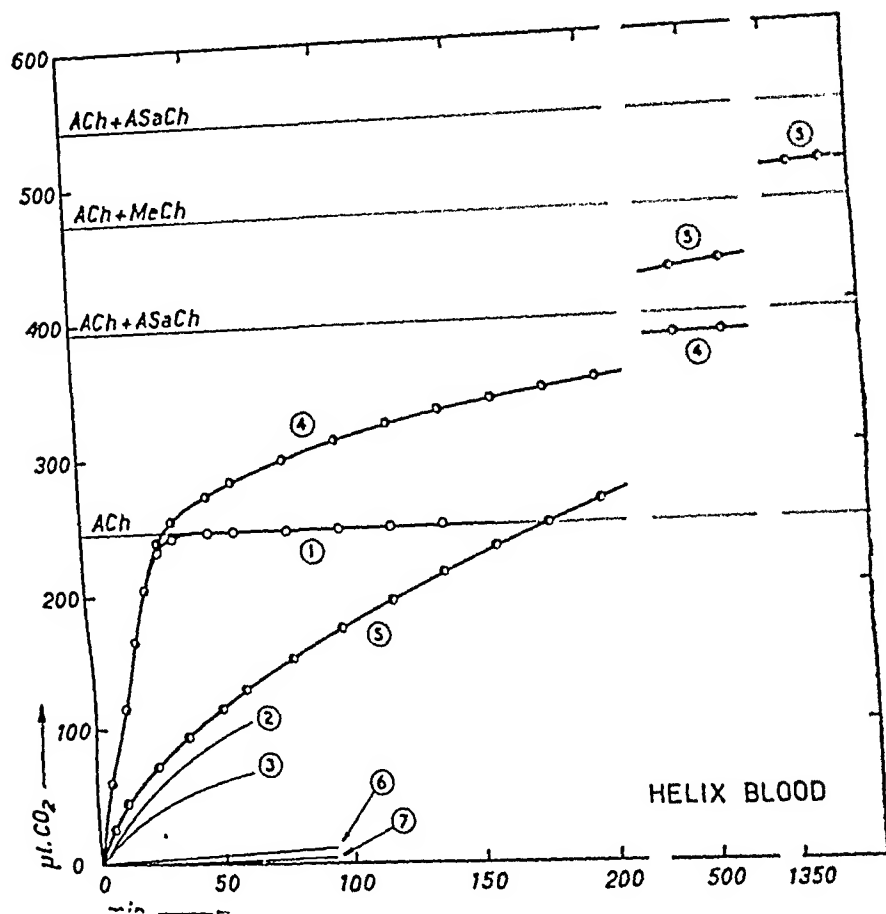


Fig. 36.

Fig. 37. Total (enzymic + non-enzymic) hydrolysis of ACh, MeCh, ASaCh, and mixtures of these substrates by *Helix* blood (50 µl.).

1. ACh; 2. MeCh; 3. ASaCh; 4. ACh + MeCh; 5. ACh + ASaCh; 6. Non-enzymic hydrolysis of ACh and ASaCh respectively; 7. Non-enzymic hydrolysis of MeCh.

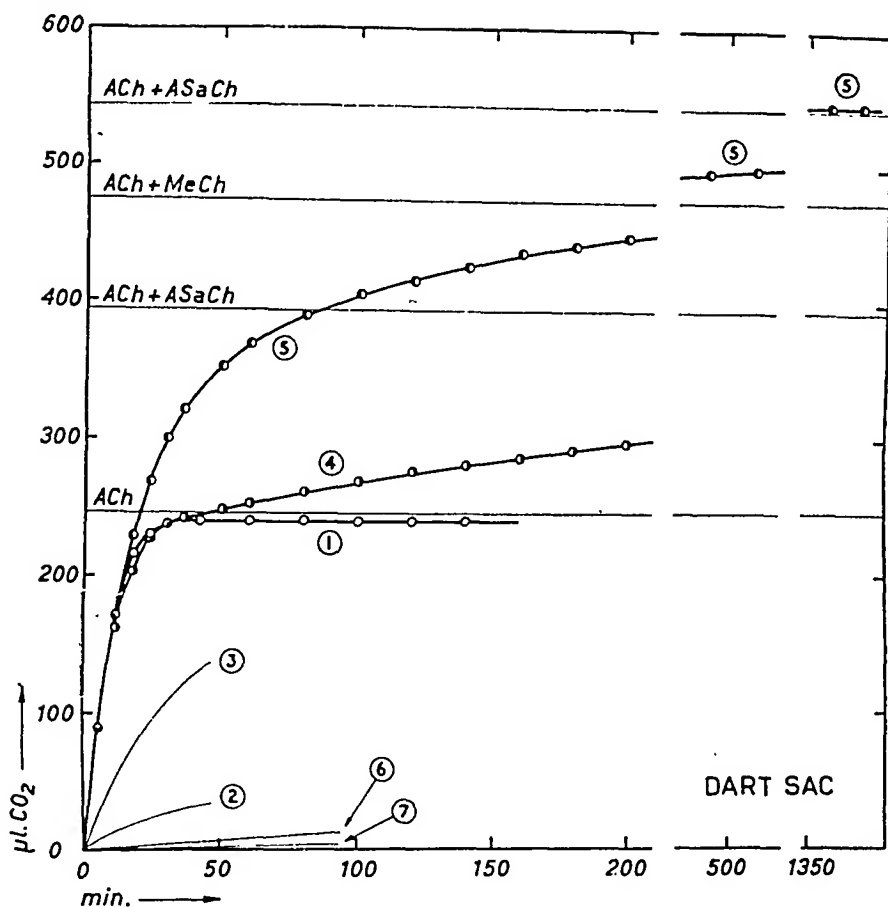


Fig. 38. Total (enzymic + non-enzymic) hydrolysis of ACh, MeCh, ASaCh, and mixtures of these substrates by the dart sac (50 mg.). Key as in Fig. 37.

Both ester linkages of ASaCh were split: in the mixture of ACh and ASaCh, when the reaction had stopped, the amount of CO_2 evolved (546 $\mu\text{l.}$) equaled almost exactly the sum of the calculated values (543 $\mu\text{l.}$) for complete destruction of ACh (246.5) and both ester linkages of ASaCh (296.5). The rate of hydrolysis was slowed down greatly with time of reaction, and it is assumed that the choline-ester linkage in ASaCh was split at a higher rate relatively to the acetyl-ester linkage, the slow breakdown of which was shown by the low rate of hydrolysis in later periods. This falling off of reaction velocity was most probably also due to the inhibiting action of choline, formed during the hydrolysis (see Chapter X).

C. SUMMARY OF CHAPTER XII

The enzymic hydrolysis of mixtures of substrates were determined. Such experiments demonstrated that in some cases a mixture of enzymes is present; in other cases one enzyme was found to split choline esters as well as non-choline esters.

Vertebrate blood plasma contains in most cases a non-specific ChE splitting choline esters and TB as well. In erythrocytes, brain, *Labrus*- and *Sepia*- liver two esterases are present, a specific ChE and an esterase acting upon TB, and, as was shown in the case of *Sepia* "liver", also ASa. In all cases ACh is split at the highest rate by the specific ChE, but it seems not always to have the highest affinity for the enzyme. Thus ASaCh has a higher affinity for the *Helix*-blood ChE than has ACh, but the first substrate is split at a relatively lower rate than ACh. A similar observation has been made for the ChE activity of *Sepia* "liver" in the hydrolysis of ACh and AAn.

CHAPTER XIII

DISCUSSION

Since the brilliant researches of OTTO LOEWI and HENRY DALE the interest in ACh has been ever growing. The rôle of ACh in nervous activity is one of the most debated problems in physiology. The chemical reactions connected with the function of the nervous system have attracted general interest and much information about this function has been obtained by studying the enzymes involved. One of these biocatalysts is cholinesterase, the extremely active enzyme system required for a rapid inactivation of free acetylcholine.

The enzymic hydrolysis of ACh was at first studied chemically using blood serum as enzyme source. When it was shown that it is unlikely that serum contains a specific ChE, other animal esterases were investigated for their specificity in splitting ACh. In these studies it was demonstrated that the ChE activities of various sources are not identical and then the hypothesis arose that two types of ACh-splitting enzymes exist.

Information about the characteristics of the ChE action, however, is not sufficiently complete to permit this "type theory" to be regarded as an exhaustive interpretation of the ChE problem. It has been shown for certain that ChE is not a single entity with identical properties irrespective of the enzyme source. The data in this paper as well as those obtained by other investigators clearly indicate this. The activity differs in a given species from tissue to tissue, as well as in a given tissue from species to species. Such differences are easily understood if we remember that the substrate specificity probably depends upon the protein component (apo-enzyme) of the enzyme molecule and that this component may greatly vary from tissue to tissue. In blood serum, for instance, the apo-enzyme presumably is an albumin; in the erythrocytes, on the other hand, where the ChE has been shown to be bound to the cell membrane, lipoproteins most probably constitute the apo-enzyme. It is supposed that this is also true for the ChE of motor end-plates, nerve fibres, ganglion cells,

and glands, and that the enzyme of the nervous system is bound to the cell structure. Hence, the blood cell ChE should be much more like the nerve esterase than the serum esterase. The data reported in this paper clearly indicate the identity of the ACh-splitting enzymes in erythrocytes and brain. This ChE has been regarded as specific for ACh which was found to be hydrolysed at a higher rate than any other ester. Apart from this "specific" ChE, an other esterase exists, *e.g.*, in serum, which hydrolyses ACh and ordinary esters (*e.g.*, tributyrin) as well. The properties of this enzyme, regarded as a "non-specific" ChE, are not similar to those of the "specific" ChE. The ChE of other tissues are in certain cases more like the "specific" ChE, in others more like the "non-specific" one, and in still others a mixture of esterases is likely to exist.

The methods proposed for distinguishing various choline-ester splitting esterases, such as the use of acetyl- β -methylcholine and benzoylcholine or other arbitrarily chosen choline esters, may be valuable in certain respects, but they would not be sufficient to support a satisfactory classification of such enzymes. It has been demonstrated above that the substrate concentration must be considered in comparing the ChE activities towards various esters. Thus the activity-substrate concentration relationship differs for a given enzyme from substrate to substrate. For this reason, one of two esters, which are acted upon by the same enzyme, may be split at a higher or lower rate than the other depending on the substrate concentration used. For instance, the "specific" ChE of erythrocytes and brain split ASaCh at a lower rate than ACh at low substrate concentrations, but at a higher rate when the substrate concentrations are high. It has also been shown that this enzyme hydrolyses the acetyl-ester linkage in ASaCh and therefore the term "specific" ChE is not adequate. In a recent paper, moreover, BODANSKY (1946) has reported that triacetin is split by this enzyme more rapidly than ACh.¹ A strict classification of choline-ester splitting enzymes therefore cannot be made on the basis of such experiments. Further criteria of ChE action must be considered in order to obtain a satisfactory picture.

Joining in the suggestion of RICHTER and CROFT (1942) and BODANSKY (1946), the present author regards the cholinesterases

¹ No information regarding the triacetin concentration used was reported. — This paper of BODANSKY came to the author's notice only when the experimental part of this work was accomplished.

as a "family" of related enzymes with widely divergent properties. The following classification of these enzymes is proposed in the light of recent findings. It is based on the activity-substrate concentration relationship for the enzymic hydrolysis of *ACh*, which, in the author's opinion, gives the most characteristic feature for making possible a satisfactory classification of such enzymes in two distinct groups. Group I is characterised by the inhibition of ChE activity at high *ACh* concentrations. The enzymes of Group II follow the MICHAELIS-MENTEN formulation which means that their activities are maximal only at infinite substrate concentration. The properties of the members within each group may then differ in certain other respects.

Group I includes the cholinesterases of the nervous system, erythrocytes, *Helix* blood, and *Sepia* "liver", studied in the present investigation. These enzymes display optimum activity at about 3×10^{-3} -M *ACh*. Triburytin is not split by them, but the specificity towards choline esters is not an absolute one. The optimum substrate concentration in the hydrolysis of *MeCh* is not always the same as in the hydrolysis of *ACh*. Clupeine causes a shift of the optimum substrate concentration in the hydrolysis of *ACh* to higher concentrations. The ChE of erythrocytes and brain are identical, but differ in certain respects from the *Helix*-blood ChE and the *Sepia*-"liver" ChE. Such differences have been observed in the actions of choline, caffeine, and quinine, and in the ability to split various esters. The *Helix*-blood ChE is characteristic of its high activity towards *AA*n. The shark-plasma ChE also belongs to Group I, but seems not to be identical with the other members of the same group.

Group II includes the choline-ester splitting esterases of certain sera (e.g., man, horse) and of the dart sac (*Helix pomatia*). These esterases also split tributyrin. In the hydrolysis of *BzCh* and *ASaCh* respectively the serum ChE activity is inhibited by excess of substrate. This is not the case with the dart-sac ChE which splits *BzCh* at a relatively low rate. Clupeine inhibits the serum ChE, but not the dart-sac enzyme. The serum ChE is competitively inhibited by choline, but not the dart-sac esterase. Quinine inhibits both types of ChE non-competitively.

It is obvious that the definition of cholinesterases ("specific" and "non-specific"), given in the General Introduction (p. 1), must be revised. As far as *ACh* is the only choline ester chemically identifiable in animal tissues, the hydrolysis of this ester may be

taken as a prototype of reactions in which ChE are catalysts. Cholinesterase may then be defined as an esterase which *at optimum conditions* hydrolyses ACh at a higher rate than any other ester. These optimum conditions in the hydrolysis of ACh are *a priori* not identical with those prevailing in the hydrolysis of other esters. When, for instance, the substrate concentration is arbitrarily chosen in the enzymic hydrolysis of a choline ester or a non-choline ester, the optimum conditions are not the same as those of the hydrolysis of ACh, the rate of reaction may be lower, the same, or even higher than that of the ACh hydrolysis. When an ester is split at a higher rate than ACh at the optimum conditions of the hydrolysis of the latter, the reaction is not catalysed by a ChE. Consequently, the high rate of BzCh hydrolysis in the action of guinea-pig liver, for instance, is *not* due to a ChE; that this reaction is not due to a specific "benzoylcholine esterase" is demonstrated above.

It is important to keep in mind the great differences in ChE activities of various sources, when elucidating the physiological significance of this enzyme. The relation between ChE activity and various pathological conditions has been studied concerning mainly the activity of blood serum. This enzyme most probably does not play any essential rôle in the regulation of nervous activity and it is not justifiable "von der ChE des *Serums* auf pathologische Vorgänge in der funktionellen ACh-Hydrolyse zu schliessen" (SCHAEFER, 1947). The variation of the serum-ChE activity, however, may possibly help in the search for factors governing the state of the serum proteins. Thus the formation of serum albumin seems to parallel that of serum ChE, the activity of which is considered as a test of liver function. Moreover, McCANCE, WIDDOWSON and HUTCHINSON (1948), in a recent paper, have reported that the level of the serum ChE is a very useful index of the state of nutrition and that alterations in diet exert a considerable influence on this activity.

It is also important to know that the ChE of erythrocytes and brain have much higher activities at low than at high ACh concentrations. Consequently, these enzymes are considerably more active at the physiological ACh concentrations than serum ChE, a fact of great value in elucidating the rôle of the ACh-ChE system. The optimum substrate concentration can be changed by adding certain substances; in the presence of the strongly basic clupeine, for instance, higher ACh concentrations are needed

to obtain optimum enzyme activity, and the negatively charged gum arabic has the opposite effect. The affinity of the positively charged ACh ion for ChE may be proportional to the negative charge of the enzyme. Hence, with increasing negativity the optimum conditions are changed to lower substrate concentration, that is, the conditions are being physiologically more suitable. The author is inclined to suppose that such a change in ChE activity occurs during nervous activity. In depolarisation, the neuronal surface becomes negative and since ChE is concentrated exclusively at this surface, the enzyme activity will be optimal at lower ACh concentrations. This possibly explains the fact, demonstrated by NACHMANSON *et al.*, that a quantitative parallelism exists between the voltage of action potential and the "concentration" of ChE. It is not necessarily the concentration, but the state of optimum conditions of ChE action, that parallels the voltage, and this change in optimum condition is merely a secondary event caused by the depolarisation of the neuronal surface.

GENERAL SUMMARY

The object of the present investigation was to compare chemically the cholinesterase activities of various animal tissues and to attempt a more general classification of such enzymes. The experimental results have been summarised at the ends of Chapters VII, IX, X, XI, XII (pp. 92, 124, 138, 147, 155). In the separate summaries more detailed information will be found.

The physiological significance of cholinesterase has been briefly discussed (Chapter I) in connection with the most prominent features of the physiological and pharmacological effects of acetylcholine and the rôle of the system acetylcholine-cholinesterase in the chemical mechanism of nervous activity. A condensed review of the literature concerning the distribution of cholinesterase in different tissues and different groups of animals has been compiled. The possible correlation between the concentration of cholinesterase and various pathological conditions has claimed considerable attention, but the mass of data obtained by various groups of investigators, in most cases, has not allowed of any conclusion as to such a relationship.

The previous literature on the chemistry of cholinesterase has been exhaustively reviewed (Chapter II), concerning the specificity, the proposed synthesising action, various methods of activity determination, units used in expressing cholinesterase activity, methods for purifying the enzyme, the chemical nature of cholinesterase, and physical and chemical factors influencing its activity.

In the present investigation the cholinesterase activity has been determined manometrically by the WARBURG technique. A detailed account of the method used has been reported (Chapter III).

The following substrates (Chapter IV) have been employed: acetylcholine chloride (also the bromide and iodide), acetyl- β -methylcholine chloride, carbaminoylcholine chloride, benzoylcholine chloride, N-acetyl-*p*-aminobenzoylcholine chloride, salicylcholine chloride, acetylsalicylcholine chloride, acetylsalicylic acid (Na-salt), tributyrin, and ethyl acetate. The enzymic hydrolysis of these substances have been compared using enzyme prepara-

tions from serum, erythrocytes, brain, muscle, liver, kidney, and intestine of various vertebrate animals, from different tissues of certain invertebrates, and from the blood of *Helix* and *Spirographis*.

A detailed study has been performed on the non-enzymic hydrolysis of the substrates as function of concentration (Chapter V). Both ester linkages in acetylsalicylcholine are split non-enzymically.

The plasma of horse, man, guinea pig, and fowl contain cholinesterase, horse plasma having the highest and fowl plasma the lowest activity (Chapter VI). The hydrolysis of benzoylcholine, salicylcholine, acetylsalicylcholine, and tributyrin run parallel to that of acetylcholine. Benzoylcholine is split at a higher rate than acetyl- β -methylcholine; the reverse holds for the cholinesterase of fowl plasma. Acetylcholine is also split by the acetylcholine-hydrolysing enzyme of plasma. Other esters are not or very little split. The plasma of cow and fish have very low cholinesterase activity.

The erythrocyte cholinesterase hydrolyses acetyl- β -methylcholine and acetylsalicylcholine at much higher rates than benzoylcholine. Tributyrin is split at a low rate by an esterase not identical with cholinesterase. Cholinesterase is bound to the cell membrane and can be precipitated together with the stroma by adding hydrochloric acid to pH 6.5 (horse). The enzyme goes into solution by treating the precipitate with lysolecithin.

The blood of *Helix pomatia* hydrolyses acetylcholine at a very high rate. The activity towards acetylcholine is greater than towards acetyl- β -methylcholine and acetylsalicylcholine. Other esters including benzoylcholine are split at low rates. The enzyme is precipitated at 50 per cent saturation with ammonium sulphate.

The blood of *Spirographis Spallanzani*, which hydrolyses acetylsalicylic acid and acetylsalicylcholine at high rates, but not the simple choline esters, is supposed to contain a "salicylesterase".

The brain cholinesterase (mammals, fish) splits acetylcholine at a higher rate than other esters, and acetyl- β -methylcholine at a higher rate than benzoylcholine. Acetylsalicylcholine is hydrolysed at a higher rate than acetyl- β -methylcholine. Other esters are not split. The hagfish brain has a low cholinesterase activity.

The fish muscles have a stronger cholinesterase activity than most mammalian muscles.

The liver-esterase activity differs greatly from animal to animal.

Esterases of high activities towards acetylcholine split in most cases acetyl- β -methylcholine at a higher rate than benzoylcholine. Guinea-pig liver hydrolyses benzoylcholine, acetylsalicylcholine, and acetylsalicylic acid at high rates, and acetylcholine at a very low rate.

Guinea-pig kidney splits acetylsalicylic acid at a higher rate than other esters studied; the choline esters are acted upon only slightly. The presence of a "salicyl-esterase" is assumed. The esterase activity of intestine (guinea pig) resembles in many ways that of kidney.

The air bladder of cod contains cholinesterase in low concentration.

The dart sac of *Helix pomatia* has a very high cholinesterase activity and is composed of smooth muscles richly supplied with nerve fibres.

The "liver" of cuttlefish (*Sepia officinalis*) contains cholinesterase with high activity towards acetylcholine.

Low cholinesterase concentrations have been found in the muscles of spider crab (*Maia squinado*), different parts of *Balanoglossus clavigerus*, and the nerve-net containing parts of the anemone *Sagartia parasitica*.

Cholinesterase activity is absent in unfertilised sea-urchin eggs (*Paracentrotus lividus*). At stage 20 hours there is a rise in activity which grows steeper as it progresses to high cholinesterase values of the stage 48 hours. The hydrolysis of acetyl- β -methylcholine parallels that of acetylcholine. Unfertilised eggs split tributyrin, acetylsalicylic acid, and acetylsalicylcholine by the action of an esterase distinct from cholinesterase. The low esterase activities of the stage 65 hours are probably due to an involution.

Bee venom has no cholinesterase activity, but inhibits horse serum cholinesterase.

The kinetics of various cholinesterase reactions vary to a great extent (Chapter VII). The enzymic hydrolysis of acetylcholine as a first-order reaction is true only under certain definite limited conditions. In most cases at relatively high substrate concentration and low cholinesterase concentration the amount of substrate hydrolysed is proportional to time within the 40—60 minute period.

Direct proportionality between reaction rate and cholinesterase concentration has usually been obtained (Chapter VIII).

A comprehensive study has been performed on the activity-substrate concentration relationships for a variety of cholin-

esterase-substrate combinations, also in a medium containing various substances (physostigmine, potassium chloride, clupeine, gum arabic) (Chapter IX). The theory of this relationship has been discussed applying the MICHAELIS-HALDANE interpretation.

Horse serum cholinesterase, acting upon acetylcholine, gives a familiar dissociation curve ($K_s = 3.2 \times 10^{-3}$). The hydrolysis of benzoylcholine and of acetylsalicylcholine are depressed by excess of substrate (optimum substrate concentration 1.0×10^{-3} M). The cholinesterase activities of erythrocytes (cow, horse) and brain (dog) have an optimum at 2.88×10^{-3} -M and 3.55×10^{-3} -M acetylcholine respectively. Also the hydrolysis of acetyl- β -methylcholine is depressed by high substrate concentrations, but acetylsalicylcholine gives familiar dissociation curves for both esterases. The cholinesterases of *Sepia* "liver" and *Helix* blood resemble those of erythrocytes and brain regarding activity-substrate concentration relationships. The cholinesterase activity of the dart sac is not depressed by high acetylcholine concentrations. Familiar dissociation curves have been obtained in the hydrolysis of benzoylcholine and acetylsalicylcholine by guinea-pig liver and of acetylsalicylcholine by cow kidney.

Physostigmine inhibits strongly the cholinesterase activity at all substrate concentrations. The hydrolysis of tributyrin by horse serum is also inhibited. Physostigmine inhibits only slightly the hydrolysis of benzoylcholine by guinea-pig liver and cow kidney, and has no influence on the tributyrinase activities of erythrocytes (cow) and brain (dog). Potassium chloride has no or very weak action on cholinesterase activity. Clupeine inhibits cholinesterase competitively and does not influence the esterases of guinea-pig liver and the dart sac. Gum arabic activates slightly the cholinesterase activity in certain cases.

Choline inhibits competitively the cholinesterase activities of horse plasma, cow erythrocytes, dog brain, and *Helix* blood (at low acetylcholine concentrations) (Chapter X). The dart-sac cholinesterase is inhibited by choline non-competitively. The enzymic hydrolysis of tributyrin is depressed very slightly in all cases studied, so also the hydrolysis of benzoylcholine and acetylsalicylcholine by guinea-pig liver and cow liver.

Methylene blue inhibits strongly cholinesterase activity in all cases (Chapter XI). Caffeine and neurine inhibit the cholinesterases of erythrocytes and brain more strongly than those of horse serum, *Helix* blood, and the dart sac. Quinine is a more potent

non-competitive inhibitor of the latter esterases than of the former; the *Helix*-blood cholinesterase is activated by quinine of low concentration. Cystine activates weakly the cholinesterases of horse serum and *Helix* blood, but this compound generally has no influence on cholinesterase activity.

Experiments with mixtures of substrates (Chapter XII) have demonstrated that the plasma of horse and guinea pig each contain a cholinesterase which splits choline esters and tributyrin as well. The cholinesterases of erythrocytes, brain, *Labrus*- and *Sepia*-liver are distinct from the esterases splitting tributyrin. Acetylsalicylcholine depresses the rate of acetylcholine hydrolysis by *Helix* blood.

The cholinesterases are regarded as a "family" of related enzymes with widely divergent properties (Chapter XIII) and defined as esterases which at optimum conditions hydrolyse acetylcholine at a higher rate than any other ester. A classification of such enzymes in two distinct groups is based on the activity-substrate concentration relationships. *Group I* is characterised by the inhibition of cholinesterase activity at high acetylcholine concentrations (optimum substrate concentration 3×10^{-3} M) and includes the esterases of the nervous system, erythrocytes, *Helix* blood, and *Sepia* "liver". The cholinesterases of brain and erythrocytes are identical, but differ in certain respects from the other members of the same group. The cholinesterases of *Group II* have maximum activity at infinite acetylcholine concentration and occur in certain sera and in the dart sac of *Helix*. The properties of these enzymes also differ in certain respects. The specificity towards choline esters is in neither group an absolute one.

The physiological significance of this new conception of acetylcholine-splitting enzymes is discussed briefly.

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